ADDENDUM

p xxii: Comment: Chapter 4 (Urmaliya *et al.*, 2010a) and Chapter 5 (Urmaliya *et al.*, 2010b) have been accepted for publication.

p 6 line 7: Comment: "Sarcoplasm contents" refer to the ionic contents (Na^+ and Ca^{2+}) of the sacroplasm (Solaini *et al.*; 2005).

p 6 line 8: delete *sarcolemmal reticulum* and read "*sarcoplasmic reticulum* Ca^{2+}-ATPase, resulting in..."

p 11: Add at the end of para 1:
"After ischemia, excessive cytosolic Ca^{2+} overload was observed in the cardiomyocytes. During initial phase of reperfusion reverse Na^+/Ca^{2+} exchange exacerbate the cytosolic Ca^{2+} overload, which causes the hypercontracture. This increased cytosolic Ca^{2+} increase the accumulation of mitochondrial Ca^{2+}, which has harmful effects on mitochondrial function (Piper *et al.*, 2004; Solaini *et al.*, 2005)."

p 15 line 12: delete "increased energy requirement" and read "Due to decreased oxygen supply under hypoxic..."

p19: Add at the end of para 1:
"Modification at the N^6-position shows the adenosine A_1 receptor selective adenosine derivatives. The oxidation of the 5`-carbon of the ribose, amidation of the resulting uronic acid and *N*-substitution of the amide is tolerated in the ribose moiety of adenosine. Removal of the 2`- and 3`-hydroxyl groups completely abolishes agonistic activity of the adenosine $A_1$ receptor agonist $N^6$-cyclohexyladenosine. Modification at the 5`-position with carboxyethylamide and substitution with ethylamide at 2`-position of purine increases the affinity for the adenosine $A_{2A}$ receptor, which lead to the development of the NECA derivative CGS 21680 (2-[*p*-(2-carboxyethyl)phenethylamino] adenosine-5`-*N*-ethyluronamide. The introduction of N^6-substituents into 5`- modified adenosine derivatives have shown a high affinity for the adenosine $A_3$ receptor. The 5`-modification in the 5`-*N*-methyluronamide is resulted in the highest potency for the adenosine $A_3$ receptor. $N^6$-benzyl-5`-*N*-methyl-carboxamideadenosine (MECA) have shown high affinity for the rat adenosine A_3 receptor. Modification at the 5`-position as uronamide and/ or as $N^6$-benzyl derivatives increases the affinity for the adenosine $A_3$ receptor. Substitution of the benzyl group with nitro and halo groups at the 3`- position mainly enhances the affinity and selectivity at the adenosine A_3 receptor."

p 21 para 2, p 93 para 1, p 126 para 2, p 155 para 1: Comment:
"The result of the present study should be interpreted with a caveat that the adenosine A_1 and A_{2A} antagonist DPCPX and ZM241385 respectively, have a relatively high binding affinity for the adenosine A_{2B} receptor as well in various rodent species, which has been reported recently (Auchampach *et al.*, 2009). The adenosine A_{2B} antagonist MRS1754 (200 nM) used in the present study has shown some binding affinity for the adenosine A_1 receptor in rat (Table 1.2 B). In hindsight, a lower concentration of DPCPX and MRS1754 would have been appropriate."

p 23: Add at the end of Table 1.2:

Table: 1.2 (B) Binding affinities of agonists and antagonists to rat (Rt), mouse (M), rabbit (R) and dog (D) adenosine receptor (AR) subtypes used in the present study (Jiang *et al.*, 1997; Fredholm *et al.*, 2001; Auchampach *et al.*, 2009).

Adenosine Receptor (AR) Subtypes		K_i value for AR (nM)			
		A_1AR	A_{2A}AR	A_{2B}AR	A_3AR
Tools agonist	A_1 CPA	0.59^{Rt}	460^{Rt}	Not available (NA)	920^{Rt}
	A_{2A} CGS21680	3100^{Rt}	22^{Rt}	NA	580^{Rt}
	A_1/ A_2 NECA	6.3^{Rt}	10^{Rt}	1001^{M}; 2060^{R}; 3001^{D}	NA
	A_3 IB-MECA	54^{Rt}	56^{Rt}	1204^{M}; 2505^{R}; 2606^{D}	1.1^{Rt}
Tools antagonist	A_1 DPCPX	0.3^{Rt}	340^{Rt}	186^{Rt}; 86.2^{M}; 96^{R}; 147^{D}	$>10{,}000^{Rt}$
	A_{2A} ZM241385	$2{,}000^{Rt}$	0.30^{Rt}	31.1^{M}; 109^{R}; 76.2^{D}	$150{,}000^{Rt}$
	A_{2B} MRS1754	17^{Rt}	610^{Rt}	12.8^{Rt}; 3.39^{M}; 1.79^{R}; 12.8^{D}	$>10{,}000^{Rt}$
	A_3 MRS1191	$40{,}000^{Rt}$	$>10{,}000^{Rt}$	NA	31.4^{Rt}

p 25 line 19: delete "activation" and read "Adenosine A_{2A} receptor deletion and antagonism reduces ROS production...in cardiac cells."

p 26 line 18: delete the sentence "and are used for the pharmacological evaluation of the adenosine A_3 receptor-mediated hemodynamic effect in isolated rat and rabbit hearts and in *in-vivo* pig heart (Lasley *et al.*, 1999)"

p 31 line 3: delete "A consequence of this is stimulation of" and read "Ischemia reperfusion stimulates intracellular calcium release from..."

p 38 line 14: delete the sentence "Lasley *et al.* (1999) reported that in isolated rat heart, Cl-IB-MECA-mediated vasodilation was blocked by the A_{2A} antagonist; SCH-58261."

p 43: Add at the end of para 2:
"Ischemia preconditioning (PreC) have shown a biphasic response of cardioprotection, in which first phase apparent immediately and last for 1-2 hrs known as classical or early ischemic PreC. After 12-24 hrs the second phase of cardioprotection appears and last for 48-72 hrs is called the "second window of protection or delayed or late ischemic PreC (Kuzuya *et al.*, 1993; Marber *et al.*, 1993; Hausenloy *et al.*, 2010). Adenosine produced during ischemic PreC (Liu *et al.*, 1991) stimulate delayed ischemic PreC by activation of PI3K-Akt, ERK1/2 and JAK, which further activates STAT1/2 and NFkB signaling to provide cardioprotection (Baxter *et al.*, 1994; Hausenloy *et al.*, 2010). Studies show that delayed ischemic PreC was abolished in the presence of the non specific adenosine receptor antagonist 8-SPT, which supports the cardioprotective role of endogenous adenosine in delayed ischemic PreC. Furthermore, the adenosine A_1 receptor agonist CCPA could enhance the cardioprotective effect of delayed ischemic PreC in *in-vivo* rabbit heart ischemia reperfusion injury (Baxter *et al.*, 1994; Dana *et al.*, 1998)."

p 46: Add at the end of Table 1.6:

References	Model / species	Preconditioning and/or treatment	Receptor involved and ligands used	Pathway info/ mechanism	Cardiopr-otection
(Liu *et al.*, 1991)	In situ and isolated Rabbit heart; 30 min I/3 hrs R	PreC- 5 min I and 10min R Agonist 15 min pre-ischemia	A_1 (R-PIA, 8-SPT), A_2 (PD115,199)	Preconditioning induce adenosine level, which activate A_1 receptor	Yes
(Thornton *et al.*, 1992)	In vivo rabbit hearts; 30 min I/3 hrs R	PreC- 5 min I and 10min R Agonist 15 min pre-ischemia or at Reperfusion	A_1 (R-PIA, CCPA), A_2 (CGS21680)	Preconditioning cardioprotection by A_1 receptor activation	Yes

(Auchampach *et al.*, 1993)	In vivo dog hearts, 60 min I/ 5 hrs R	PreC- 5 min I	A_1 (DPCPX), K_{ATP} antagonist (Glibenclamide)	Preconditioning cardioprotection by A_1 receptor activation involves opening of K_{ATP} channels	Yes

p 48 line 10: delete the sentence "Adenosine administered at the..." and read "Adenosine administered at the start of reperfusion or both before ischemia and during reperfusion has been shown to improve post-ischemic dysfunction by the activation of the adenosine A_1 receptors (Donato *et al.*, 2003)."

p 48 line 12: delete the sentence "A number of studies...in mouse hearts" and read "A number of studies have shown that activation of A_1 receptors by exogenous agonists during reperfusion in isolated rat hearts (Finegan *et al.*, 1996; Lozza *et al.*, 1997; Butcher *et al.*, 2007) or transgenic A_1 receptors overexpression in isolated mouse heart (Matherne *et al.*, 1997) minimizes ischemic contracture and cardiac dysfunction."

p 49: Add at the end of para 1:
"Reports suggest that the activation of 'reperfusion injury salvage kinases' (RISK) such as ERK1/2, p38, p54JNK are not involved in the ischemia preconditioning (Heusch 2009) or postconditioning cardioprotection (Skyschally *et al.*, 2009; Musiolik *et al.*, 2010) in the anesthetized pig ischemia reperfusion injury model. Alternative signaling pathway such as JAK-STAT pathway in the ischemia preconditioning cardioprotection has been reported (Lecour *et al.*, 2005a; Lecour *et al.*, 2005b). Ischemia postconditioning is mediated by SAFE pathway (Survivor Activating Factor Enhancement), which involves the activation of cytokine TNF-α and STAT-3 (Signal Transducer and Activator of Transcription-3) have been reported (Lacerda *et al.*, 2009)."

p 74: Comment: Adenosine A_1 and A_3 antagonist (DPCPX and MRS1191 respectively) used at the concentration of 10^{-6} M.

p 74: para 1: Comment: In cell based assay separate 4 replicate experiments were performed in triplicates for each treatment protocol.

p 92 line 9: delete "suggest that our SI model caused reversible ischemic damage" and read "suggesting that our SI model caused some degree of cell death that could be reversed by the use of an A_1 agonist."

p 104 line 17: delete the sentence "Lasley et al (1999) reported that in isolated rat heart Cl-IB-MECA mediated vasodilation was blocked by the A_{3A} antagonist SCH-58261."

p 145: Figure 4.3 Y-axis label: delete "% change from baseline" and read "% of baseline".

p 154: Add at the end of para 1:
"An exception to the requirement for A_2 receptor activation for full A_1 protection was found in the case of contracture: the rise in end diastolic pressure that occurs during ischemia. A_1-mediated reduction in contracture is dependent upon mitochondrial K_{ATP} channels (Headrick *et al.*, 2000). CPA produced a significant reduction in EDP both during ischemia (i.e. prior to CPA treatment) and at the end of reperfusion in CPA-treated A_{2A}KO mice compared to A_{2A} KO control mice, as well as in CPA treated WT mice compared to control. The reduced EDP during ischemia in this group is a finding difficult to explain; perhaps a consequence of several outlier data points in the control group. Nonetheless, the results of this study show no evidence of the requirement for A_2 receptor activation in A_1-mediated reductions in contracture."

p 219: Add into the bibliography

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Musiolik, J, van Caster, P, Skyschally, A, Boengler, K, Gres, P, Schulz, R and Heusch, G (2010) Reduction of infarct size by gentle reperfusion without activation of reperfusion injury salvage kinases in pigs. *Cardiovasc Res* 85(1): 110-117.

Skyschally, A, van Caster, P, Boengler, K, Gres, P, Musiolik, J, Schilawa, D, Schulz, R and Heusch, G (2009) Ischemic Postconditioning in Pigs: No Causal Role for RISK Activation. *Circ Res* 104(1): 15-18.

Urmaliya, V B, Pouton, C W, Devine, S M, Haynes, J M, Warfe, L, Scammells, P J and White, P J (2010a) A novel highly selective adenosine A1 receptor agonist VCP28 reduces ischemia injury in a cardiac cell line and ischemia-reperfusion injury in isolated rat hearts at concentrations that do not affect heart rate. *J Cardiovasc Pharmacol* 56(3): 282-292.

Urmaliya, V B, Pouton, C W, Ledent, C, Short, J L and White, P J (2010b) Cooperative cardioprotection via adenosine A1 and A2A receptor agonism in ischemia-reperfused isolated mouse heart. *J Cardiovasc Pharmacol* Publish Ahead of Print: 10.1097/FJC.1090b1013e3181f1003d1005.

ADENOSINE RECEPTOR-MEDIATED CARDIOPROTECTION

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

By

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April, 2010

Dedicated to my beloved family:

My Parents, Mummy Smt Urmila and Papa Shri Badri Prasad Urmaliya

Ajay Bhaiya and Pooja Bhabhi, Gaurav and Archana Bhabhi

My wife Sandhya, Sisters Anju and Shashi

Dearest Yash Pratyush (Betu) and Ishaan (Ishu)

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Summary:

Ischemic heart disease is the most common type of cardiovascular disease. The Australian Institute of Health and Welfare reported that there were 22,983 deaths (17% of all deaths in Australia) due to ischemic heart disease that were recorded in 2006. Substantial evidence accumulated from human data and animal models suggests that adenosine released under conditions of physiological stress such as hypoxia and ischemia-reperfusion injury plays an important cardioprotective role via activation of adenosine receptors (four subtypes: A_1, A_{2A}, A_{2B} and A_3) and reduces the extent of cardiac cell death. The present thesis describes a body of work investigating cardioprotection induced by adenosine receptor and synergistic cooperative interactions between adenosine A_1 and A_2 receptors during ischemia-reperfusion injury using a cardiac cell ischemia and Langendorff perfused isolated mouse/rat heart models.

Chapter 2 describes the development of a suitable pharmacological model to mimic the *in-vivo* ischemic condition using H9c2(2-1) cardiac cells. This was used for preliminary pharmacological evaluation of novel compounds synthesized by the Department of Medicinal Chemistry in our faculty. Exposure of cardiac cells to ischemic buffer for 12 hrs significantly increased the number of nonviable cells, the effect reduced in the presence of adenosine A_1 and A_3 receptor agonists N^6cyclopentyladenosine (CPA) and N^6-(3-Iodobenzyl)adenosine-5′-N-methyluronamide (IB-MECA), and novel adenosine A_1 and A_3 agonists VCP28, VCP102 and VCP103, VCP438, VCP439, VCP485, VCP486, VCP487 respectively. A novel, highly selective adenosine A_1 partial agonist VCP28 have shown the

cardioprotection at lower nanomolar concentration, which is further investigated in the isolated rat heart model in chapter 5.

Chapter 3 demonstrate the interactions between A_1 and A_2 receptors in the presence and absence of endogenous adenosine in a cardiac cell ischemia model. In a cardiac cell ischemia model necrosis was assessed using propidium iodide (5μM) staining or apoptosis using AnnexinV-PE flowcytometry. CPA (100 nM) and IB-MECA (100 nM) reduced the proportion of non-viable cells to 30.87 ± 2.49% and 35.18 ± 10.30% respectively (% of SI group, $P<0.05$, n=3-5). In the absence of endogenous adenosine, using the introduction of adenosine deaminase into the media, the protective effect of CPA was reduced, whilst the efficacy of IB-MECA was unchanged. The protective effects of CPA and IB-MECA were abrogated in the presence of their respective antagonists DPCPX and MRS1191, whilst A_{2A} and A_{2B} agonists had no significant effect. CPA-mediated protection was abrogated in the presence of both A_{2A} (ZM241385) and A_{2B} (MRS1754) antagonists. In the absence of endogenous adenosine significant protection was observed with CPA in presence of A_{2A} or A_{2B} agonists (CGS21680 or LUF5834). Apoptosis was not significantly reduced by CPA or IB-MECA. The data demonstrate that cardioprotection induced by adenosine A_1 receptor agonist in a cardiac cell ischemia model involves co-operative activation of adenosine A_{2A} and A_{2B} receptors by endogenous adenosine.

Chapter 4 looks at the synergistic cooperative interaction between adenosine A_1 and A_2 receptors in the intact heart – in ischemia-reperfused isolated mouse hearts when CPA was given briefly at reperfusion. Adenosine A_{2A} receptor knockout (A_{2A}KO) and wild-type

(WT) mouse hearts (n=9-11) were subjected to global ischemia (30 min) and reperfusion (60 min) in presence of CPA or CGS21680 for the initial 15 min of reperfusion. In WT hearts, CPA (100 nM) significantly ($P<0.05$) improved contractility (dP/dt_{max}, 52.69 ± 6.22 vs. 23.94 ± 4.93% of baseline), left ventricular developed pressure (LVDP), end diastolic pressure (EDP), reduced infarct size (7.86 ± 1.73 vs. 23.94 ± 6.62% area at risk), decreased lactate dehydrogenase efflux and increased pERK1/2 signalling. ZM241385 and MRS1754 abolished CPA-mediated cardioprotection in WT groups, similar to the DPCPX. In A_{2A}KO hearts, CPA did not improve functional recovery and pERK1/2 signaling. In this clinically relevant model of pharmacological intervention, pERK1/2-dependent A_1-mediated cardioprotection requires a cooperative activation of A_2 receptors, presumably via endogenous adenosine.

Chapter 5 describes the further evaluation of compound identified in chapter four, the novel, highly selective adenosine A_1 receptor partial agonist N^6-(2,2,5,5-tetramethylpyrrolidin-1-yloxyl-3-ylmethyl)adenosine (VCP28) in a cardiac cell ischemia model and in an isolated rat heart ischemia-reperfusion model. In the cardiac cell ischemic model, CPA and VCP28 (100 nM) treatment during ischemia significantly reduced the proportion of nonviable cells (30.88 ± 2.49, 16.17 ± 3.77% of SI group, respectively, $P<0.05$, n= 5-6) and lactate dehydrogenase efflux. In isolated rat hearts, CPA and VCP28 treatment briefly at reperfusion significantly (n= 6-8, $P<0.05$) improved postischemic contractility (81.69 ± 10.96, 91.07 ± 19.87 % of baseline, respectively), LVDP, EDP and reduced infarct size. DPCPX abolished the cardioprotective effects of CPA and VCP28 in cardiac cell ischemia and isolated rat hearts models. The data described in this chapter

demonstrate that the A_1 partial agonist VCP28 has equal cardioprotective effects to the full agonist CPA at concentrations that have no effect on isolated rat heart rate.

In conclusion, studies described in the present thesis provide compelling evidence that endogenous adenosine released during ischemic condition increase cardioprotection of A_1 agonist, and cooperatively activate adenosine A_{2A} and A_{2B} receptors in a cardiac cell ischemia and isolated mouse heart ischemia reperfusion models. Of note, the synergistic "cooperative A_1 and A_2 ischemic protection" involve downstream activation of ERK1/2 phosphorylation signalling in isolated mouse hearts. Novel and highly selective adenosine A_1 and A_3 receptor agonists were screened and the A_1 partial agonist VCP28 was shown to provide cardioprotection when administered during ischemia (pharmacological preconditioning) in a cardiac cell ischemia model and during reperfusion (pharmacological postconditioning) with greater efficacy and potency than the same compound was able to activate pathways mediating acute cardiac effects, such as minimal bradycardic effect, in isolated rat heart model. This thesis demonstrates that adenosine A_1 receptor-mediated cardioprotection during ischemia-reperfusion damage is dependent on cooperative activation of adenosine A_2 receptor by endogenous adenosine.

Declaration:

I hereby declare that the research work described in this thesis is my own original work and no portion of this thesis has been submitted for the award of a degree or diploma at this or any other university or other equivalent institution. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

(Signed)

Vijay Urmaliya **Date:** 19th April 2010

Publications and Communications:

Publications arising from work presented in this thesis

Urmaliya, VB, Church, JE, Coupar, IM, Rose'Meyer, RB, Pouton, CW, White, PJ (2009) Cardioprotection induced by adenosine A_1 receptor agonists in a cardiac cell ischemia model involves cooperative activation of adenosine A_{2A} and A_{2B} receptors by endogenous adenosine. *J. Cardiovasc. Pharmacol.* **53**(5)**:** 424-433.

Urmaliya VB, Pouton CW, Ledent C, Short JL, White PJ (2010) Cooperative cardioprotection via adenosine A_1 and A_{2A} receptor agonism in ischemia-reperfused isolated mouse heart. *J Cardiovasc Pharmacol,* **doi:10.1097/FJC.0b013e3181f03d05.**

Urmaliya VB, Pouton CW, Devine SM, Haynes JM, Warfe L, Scammells PJ, White PJ (2010) A novel, highly selective adenosine A_1 receptor agonist VCP28 reduces ischemia injury in a cardiac cell line and ischemia-reperfusion injury in isolated rat hearts at concentrations that do not affect heart rate. *J Cardiovasc Pharmacol,* **56**(3): 282-292.

Gregg A, Bottle SE, Devine SM, Figler H, Linden J, White P, Pouton CW, **Urmaliya V**, Scammells PJ (2007) Dual acting antioxidant A_1 adenosine receptor agonists. *Bioorg Med Chem Lett.* **17**(19)**:** 5437-5441.

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Communications:

Part of the work in thesis was presented as oral presentations at the following conferences:

Urmaliya V, Pouton C, Coupar I, Ledent C, Short J, White P, "A cooperative cardioprotective effect of adenosine A_1 and A_{2A} receptor agonism in ischemia-reperfusion damage in the isolated perfused mouse heart", *Proceedings of 43rd Australasian Society of Clinical & Experimental Pharmacologist and Toxicologist (ASCEPT) Annual Scientific Meeting 2009*, Sydney Convention and Exhibition Centre, Sydney, Australia, Nov 29- Dec 2, 2009.

Urmaliya V, Pouton C, Coupar I, Ledent C, Short J, White P, "A cooperative cardioprotective effect of adenosine A_1 and A_{2A} receptor agonism in ischemia-reperfusion damage in the isolated perfused mouse heart", *Proceedings of 4th Annual Postgraduate Research Symposium*, Faculty of Pharmacy and Pharmaceutical Sciences, Monash University, Parkville, Victoria, Australia, 30th September 2009.

Urmaliya V, Pouton C, Coupar I, Ledent C, Short J, White P, "A cooperative cardioprotective effect of adenosine A_1 and A_{2A} receptor agonism in ischemia-reperfusion damage in the isolated perfused mouse heart", *ASCEPT- Cardiovascular Special Interest Group Regional Student-Supervisor Research Day*, Baker IDI Heart and Diabetes Institute, Melbourne, 28th September 2009.

Urmaliya V, Church J, Coupar I Pouton C and White P, "Cardioprotection via adenosine A_1 receptor agonists in a cardiac cell ischemia model involves a co-operative effect of

endogenous adenosine via adenosine A_{2A} and A_{2B} receptors." *Proceedings of 3rd Annual Postgraduate Research Symposium*, Faculty of Pharmacy and Pharmaceutical Sciences, Monash University, Parkville, Victoria, Australia, 1st October 2008.

Urmaliya V, Pouton C, Coupar I, Scammells P and White P, "Cytoprotection with an Adenosine A_1 Receptor Antagonist Compared to an A_3 Receptor Agonist in a Cardiomyocyte Model of Ischemia." *14th World Congress on Heart Disease*, *Toronto, Ontario, Canada,* 26-29th July 2008; *The Journal of Heart Disease* (2008)*;* **6**(1): 70.

Part of the work in thesis was presented as posters at the following conferences:

Urmaliya V, Pouton C, Coupar I, Ledent C, Short J, White P, "A cooperative cardioprotective effect of adenosine A_1 and A_{2A} receptor agonism in ischemia-reperfusion damage in the isolated perfused mouse heart", *Proceedings of British Pharmacological Society Winter Meeting 2009*, The Queen Elizabeth II Conference Centre, *London, United Kingdom,* 15-17 December, 2009.

Urmaliya V, Pouton C, Coupar I, Ledent C, Short J, White P**,** "A cooperative cardioprotective effect of adenosine A_1 and A_{2A} receptor agonism in ischemia-reperfusion damage in the isolated perfused mouse heart", *Proceedings of Cancer Therapeutics CRC Annual Postgraduate Research Symposium*, Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia, 29th September 2009. **(Awarded Second Prize)**

Urmaliya V, Church J**,** Coupar I Pouton C and White P, "Cardioprotection via adenosine $_{A1}$ receptor agonists in a cardiac cell ischemia model involves a co-operative effect of

endogenous adenosine via adenosine A_{2A} and A_{2B} receptors" *Proceedings of* 4^{th} *Australian Health and Medical Research Congress (AHMRC)*, Brisbane, Queensland, Australia, 1^{6th} - 2^{1st} November 2008. **(Finalist for Percy and Whelan Prize)**

Urmaliya V, Pouton C, Coupar I, Scammells P and White P**, "**Effect of Endogenous Adenosine on Adenosine A_1 and A_3 Receptor Subtypes during Simulated Ischemia" *Proceedings of* 9^{th} *World Conference on Clinical Pharmacology and Therapeutics*, *Quebec, Canada,* 27^{th} July -1^{st} August, 2008.

Urmaliya V, Pouton C, Coupar I, Scammells P and White P, "Characterization of adenosine A_1 and A_3 receptors-mediated protective effect in simulated ischemia-induced necrosis in H9c2 (2-1) cardiomyocyte" *Proceedings of Australasian Society of Clinical & Experimental Pharmacologist and Toxicologist (ASCEPT)- Southeast Asian Western Pacific Regional Meeting of Pharmacologist (SEAWP)*, Adelaide, 3-6 December 2007.

Urmaliya V, Pouton C, Coupar I, Scammells P and White P, "Characterization of adenosine A_1 and A_3 receptors (ARs)-mediated protective effect in simulated ischemia-induced necrosis in H9c2 (2-1) cardiomyocyte" *2nd Annual Postgraduate Research Symposium*, Victorian College of Pharmacy, Monash University, Parkville, Victoria, Australia, 23 October 2007.

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Abbreviations:

A_{2A}KO	Adenosine A_{2A} receptor knockout
AAR	Area at risk
ADA	Adenosine deaminase
ADA	Adenosine deaminase
AF	Atrial fibrillation
AIHW	The Australian Institute of Health and Welfare
AMP579	1S-[1a,2b,3b,4a(S*)]-4-[7-[[1-[(3-chloro-2-thienyl)methylpropyl]propyl-amino]-3H-imidazo[4,5-b]pyridyl-3-yl]-N-ethyl-2,3-dihydroxycyclopentane carboxamide
AV	Atrioventricular
cAMP	Cyclic adenosine monophosphate
CCPA	2-chloro-N^6-cyclopentyladenosine
CGS21680	4-[2-[[6-Amino-9-(N-ethyl-b-D-ribofuranuronamidosyl)-9H–purin-2-yl]amino]ethyl]benzenepropanoic acid
CHA	N^6-cyclohexyl adenosine
CK	Creatinine phosphokinase
Cl-IB-MECA	2-chloro-*N*^{6}-(3-iodobenzyl)adenosine-5'-N-methylcarboxamide
COX-2	Cyclooxygenase-2
CPA	N^6-Cyclopentyladenosine
CSC	1,3,7-Trimethyl-8-(3-chlorostyryl)xanthine
cTn- I	Cardiac troponin I
cTn-T	Cardiac troponin T

DAG	Diacylglycerol
DMPA	N^6-[2-(3,5-Dimethoxyphenyl)-2-(2-methylphenyl)-ethyl]adenosine
DMSO	Dimethyl sulfoxide
dP/dt_{max}	Maximum contractility
dP/dt_{min}	Minimum contractility
DPCPX	8-Cyclopentyl-1,3-dipropylxanthine
e-5'-NTase/CD73	Ecto-5'-nucleotidase
EDP	End diastolic pressure
eNTPD/CD39	Ecto-nucleoside triphosphate diphosphohydrolase
e-PDE	Ecto-phosphodiesterase
ERK1/2	Extracellular signal-regulated kinase 1/2
GPCR	G protein coupled receptor
GPX	Glutathione peroxidase
HE-NECA	2-(1-Hexyn-1-yl)adenosine-5′-N-ethyluronamide,2-Hexynyl-5′-ethylcarboxamidoadenosine
HIF-1	Hypoxia inducible factor-1
I/R injury	Ischemia/reperfusion injury
IB-MECA	N^6-(3-Iodobenzyl) adenosine-5'-N-methyluronamide
IHD	Ischemic heart disease
iNOS	Inducible nitric oxide synthase
IP_3	Inositol-1-4-5-triphosphate ()
K_{ATP}	Potassium ATP channel
LDH	Lactate dehydrogenase

LUF5834	2-amino-4-(4-hydroxyphenyl)-6-(1*H*-imidazol-2-ylmethylsulfanyl)pyridine-3,5-dicarbonitrile
LVDP	Left ventricular developed pressure
MAP kinase	Mitogen activated protein kinase
MDA	Malondialdehyde
mitoK$_{ATP}$	ATP dependent mitochondrial potassium channel
mPTP	Mitochondrial permeability transition pore
MRS1191	3-Ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate
MRS1754	8-[4-[((4-Cyanophenyl)carbamoylmethyl)oxy]phenyl]-1,3-di(n-propyl)xanthine
NECA	5'-N-Ethylcarboxamidoadenosine1-(6-Amino-9H-purin-9-yl)-1-deoxy-N-ethyl-b-D-ribofuran uronamide
NFκB	Nuclear factor kappa B
NO	Nitric oxide synthase
OH$^{\bullet}$	Hydroxyl radical
PARP	Poly (ADP-ribose) polymerase
PI	Propidium iodide
PI3K	Phosphoinositol-3 kinase
PIA	N^6-(phenyl-2R-isopropyl)-adenosine
PKA	Protein kinase A
PKC	Protein kinase C
PLA_2	Phospholipase A_2

PLC	Phospholipase C
PostC	Postconditioning
PreC	Preconditioning
PSVT	Paroxysmal supraventricular tachycardia.
RISK pathways	Reperfusion injury salvage kinase pathways
$RO^{\bullet}$	Alkoxy radical
$ROO^{\bullet}$	Peroxy (radicals)
ROS	Reactive oxygen species
RPIA	R-N^6-(methyl-2-phenylethyl)adenosine
SA	Sinoatrial
SAH	S-adenylhomocysteine
SCH58261	7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine
sGOT	Serum glutamic oxaloacitic transaminase
SI	Simulated ischemia
SOD	Superoxide dismutase
TTC	2,3,5-triphynyltetrazolium chloride
VCP103	N-Methyl-N^6-[4-[2-[3,5-di-tert-butyl-4 hydroxybenzamido]ethyl]phenyl]adenosin-5′-uronamide)
VCP28	N^6-(2,2,5,5-tetramethylpyrrolidin-1-yloxyl-3-ylmethyl)adenosine
VCP438	N-Methyl-N^6-[4-[2-[3,5-di-*tert*-butylbenzamido]ethyl]phenyl]adenosin-5′-uronamide

VCP439	N-Methyl-N^6-[4-[2-[3,4,5-trimethoxybenzamido]ethyl]phenyl]adenosin-5′-uronamide
VCP485	N-Methyl-N^6-[4-[2-[3,4,5-trimethoxybenzamido]ethyl]benzyl]adenosin-5′-uronamide
VCP486	N-Methyl-N^6-[4-[2-[3,5-di-*tert*-butyl-4-hydroxybenzamido]ethyl]benzyl]adenosin-5′-uronamide
VCP487	N-Methyl-N^6-[4-[2-[3,5-di-*tert*-butylbenzamido]ethyl]benzyl]adenosin-5′-uronamide
VSMC	Vascular smooth muscle cells
WHO	World Health Organisation
WT	Wild type
ZM241385	4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-lamino]ethyl)phenol.

"Science is the century-old endeavor to bring together by means of systematic thought the perceptible phenomena of this world into as thorough-going an association as possible. To put it boldly, it is the attempt at a posterior reconstruction of existence by the process of conceptualization. Science can only ascertain what is, but not what should be, and outside of its domain value judgments of all kinds remain necessary". ***~Albert Einstein***

CHAPTER 1

Introduction

The present thesis describes a body of work investigating adenosine receptor activation in ischemia-reperfusion injury, utilizing *in-vitro* and *ex-vivo* models of ischemic cardiomyocytes. Ischemia-reperfusion injury is a clinical condition where myocardial injury (or infarction) is caused due to blockage and reopening (transient or sustained) of blood flow through the coronary arteries of the heart. Substantial evidence accumulated from human data and animal models suggest that adenosine plays an important protective role in ischemic-reperfusion injury and reduces the extent of cell death. Adenosine exerts its protective effect via various adenosine receptors. The cardioprotective mechanism of adenosine involves activation of various downstream signalling pathways such as extracellular signal-regulated kinase (ERK) 1/2, phosphoinositol-3 kinase (PI3K-Akt), protein kinase A (PKA) and protein kinase C (PKC). The accumulated body of knowledge distinguishing the role of synergistic interaction between adenosine receptor subtypes for cardioprotection is currently very limited. Protection demonstrated by ischemia-reperfusion injury via adenosine receptor activation is the main focus of this thesis.

1.1. Epidemiology and burden of ischemic heart disease

Ischemic heart disease (IHD) or myocardial ischemia is the most common type of heart disease. It is one of the major causes of death and disability in the world, and incurs massive financial costs to society. According to the World Health Organisation (WHO), ischemic heart disease claims 17.1 million lives every year worldwide (WHO 2009) and it is predicted that IHD could be the major cause of death in the world by year 2020 (Murray *et al.,* 1997; Lopez *et al.,* 1998). Various data suggests, that of 32 million heart attacks

worldwide, 12.5 million are fatal, and the mortality rate for heart attack victims before reaching hospital is significantly high at 40–75% (WHO 2002). The Australian Institute of Health and Welfare (AIHW) reported that the prevalence of ischemic heart disease in Australia was 637,900 for 2004–05 (AIHW 2009). Ischemic heart disease has become a huge burden on society, a fact supported by hospitalisation in 162,328 cases between years 2006–07. There were 22,983 deaths (17% of all deaths in Australia) due to IHD that were recorded in 2006 (AIHW 2009). Therefore, it is essential to comprehend the pathophysiology of IHD in order to develop therapeutic strategies and targets to be used in clinical situations.

1.2. Myocardial ischemia and ischemia-reperfusion injury

1.2.1. General definitions

"Myocardial ischemia-reperfusion injury is a medical emergency caused by loss of oxygen to the heart due to either a blockage of blood flow or reduced blood supply"

Ischemia is the condition suffered by tissues and organs when deprived of blood flow, mostly due to the effects of inadequate nutrients and oxygen, resulting in an imbalance between oxygen demand and supply. Insufficient blood supply to cardiac tissue can be due to various reasons, including increased myocardial substrate demand and blockade or narrowing of coronary artery. Ischemia develops within minutes of thrombosis or embolism forming between two affected zones i.e. the infarct core and area at risk, leading to infarction then death of a cardiac cell. Myocardial ischemia is a pathological condition that

can manifest as four clinical forms: silent ischemia, angina pectoris, variant angina and myocardial infarction (Figure 1.1) (Gasser *et al.*, 1994).

Reperfusion injury refers to the damaging effects from restoration of blood flow after more than ten minutes of ischemia, and is typically more damaging than ischemia itself due to release of inflammatory mediators and free-radicals into the heart. Ischemia-reperfusion injury leads to different clinical conditions including myocardial necrosis, arrhythmia, myocardial stunning, and endothelial/microvascular dysfunction (Moens *et al.*, 2005).

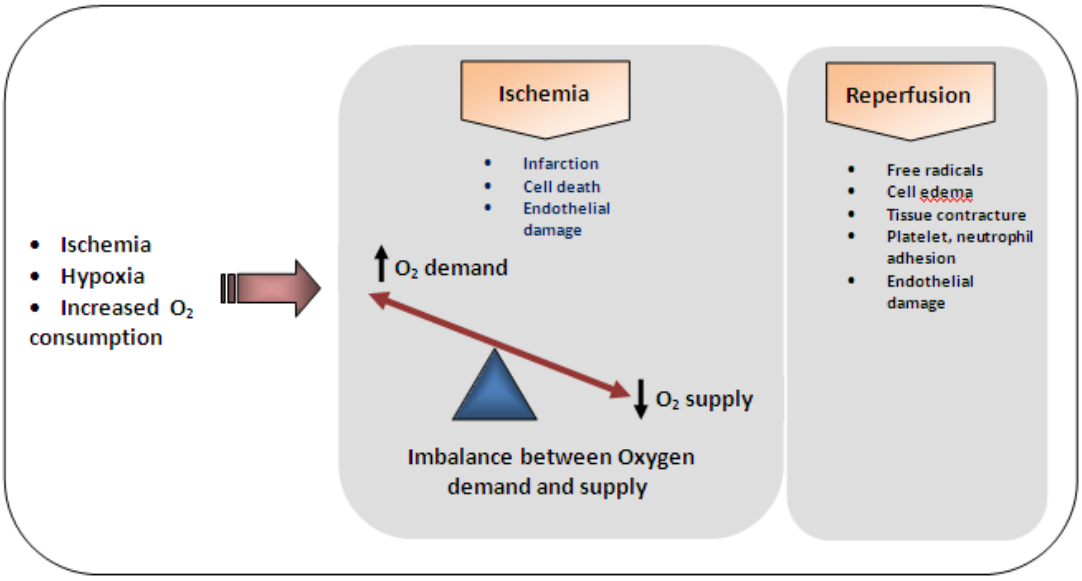


Figure 1.1 Consequences of myocardial ischemia-reperfusion injury.

1.2.2. Pathophysiology of myocardial ischemic injury

Myocardial ischemic injury involves various pathophysiological events. A large body of evidence suggests that reactive oxygen species (ROS) production increases during reperfusion injury(Kukreja *et al.,* 1997; Piper *et al.,* 1998; Ceconi *et al.,* 2003; Marczin *et al.,* 2003; Becker 2004). During ischemia various intracellular sites may generate ROS, including mitochondria by activation of mitochondrial oxidative phosphorylation, xanthine oxidase, NADH/NADPH oxidase, lipoxygenase, and activated phosphate and arachidonic acid metabolism (Ferrari *et al.,* 1993; Vanden Hoek *et al.,* 1997; Ferdinandy *et al.,* 2003) . Metabolic imbalance due to myocardial infarction leads to an increased level of harmful biochemical products and also reduces energy generation (de Leiris *et al.*, 1990). During the ischemic period, mitochondria generate free radicals from various intracellular sites (de Leiris *et al.*, 1990; Vanden Hoek *et al.*, 1997).

During normal physiological conditions cardiac cells generates adenosine triphosphate (ATP) to meet the energy demand of the organ (Carvajal *et al.*, 2003). The heart acts as a pump that uses chemical energy to generate mechanical work. Cardiac muscles generate energy by the oxidation of carbon sources (free fatty acids) together with oxygen. These fuels are mainly provided by the coronary blood flow (Carvajal *et al.*, 2003; Solaini *et al.*, 2005). Within the cardiac cells the mitochondria generate the bulk of the cardiac energy by oxidative phosphorylation and ATP production (Solaini *et al.*, 2005). Under anaerobic (ischemic) conditions, when blood flow and oxygen levels decrease, oxidative metabolism is altered to glycolytic metabolism (Carvajal *et al.*, 2003). The rapid depletion of ATP to adenosine diphosphate (ADP), adenosine monophosphate (AMP) and adenosine causes

accumulation of adenosine in the tissue. The ischemic myocardium loses its ability to sustain the negative resting membrane potential (Braasch *et al.,* 1968; Jennings 1969). During ischemia, metabolic changes occurs in the tissue, which increases the level of lactate, H^+ ions, phosphate, potassium, and pyruvate as an accumulating by-product of cellular metabolism in ischemic cardiomyocytes (Braasch *et al.,* 1968; Swynghedauw 1999; Buja 2005).

Marked changes in the sarcoplasm content have been observed during ischemia refer to the ionic content of the sarcoplam. Low ATP concentration leads to the inactivation of sarcolemmal Na^+/K^+-ATPase and sarcoplasmic reticulum Ca^{2+}-ATPase, resulting in an increase of cytoplasmic Na^+ and Ca^{2+} concentrations (Piper *et al.*, 2004; Solaini *et al.*, 2005). Mitochondrial calcium increase further develops ischemic contracture and ultimately necrosis of the cardiac myocyte. Due to the accumulation of inorganic phosphate (H_2PO_4) and metabolites, osmotic pressure of the cardiac cell increases, leading to increased K^+ efflux. During ischemia reduced ATP levels inhibit Na^+/K^+-ATPase activity which further increases the efflux of K^+ and influx of Na^+, Cl^- and water into the cardiomyocyte which causes cell swelling (oncosis; Figure 1.2). Ischemic cardiomyocytes exhibit ultrastructural changes including mitochondrial and sarcoplasmic reticulum swelling and nuclear chromatin condensation (apoptosis) (Buja 2005). Due to irreversible damage to the mitochondria and the cell membrane, various plasma markers such as lactate dehydogenase (LDH), creatinine phosphokinase (CK), cardiac troponin T (cTn-T) and I (cTn- I) and serum glutamic oxaloacitic transaminase (sGOT) are released into the blood, and the

plasma levels of these enzymes can be used to evaluate necrotic damage in the clinical situation.

The ischemic cell response to oxygen deprivation leads to the activation of hypoxia inducible factor-1 (HIF-1). Phosphorylation of HIF-1 activates it, and HIF-1 acts as a substrate for various survival kinase pathways including the MAP kinase (ERK1/2), PI-3K, p38 and JNK pathways (Lopez-Neblina *et al.*, 2006).

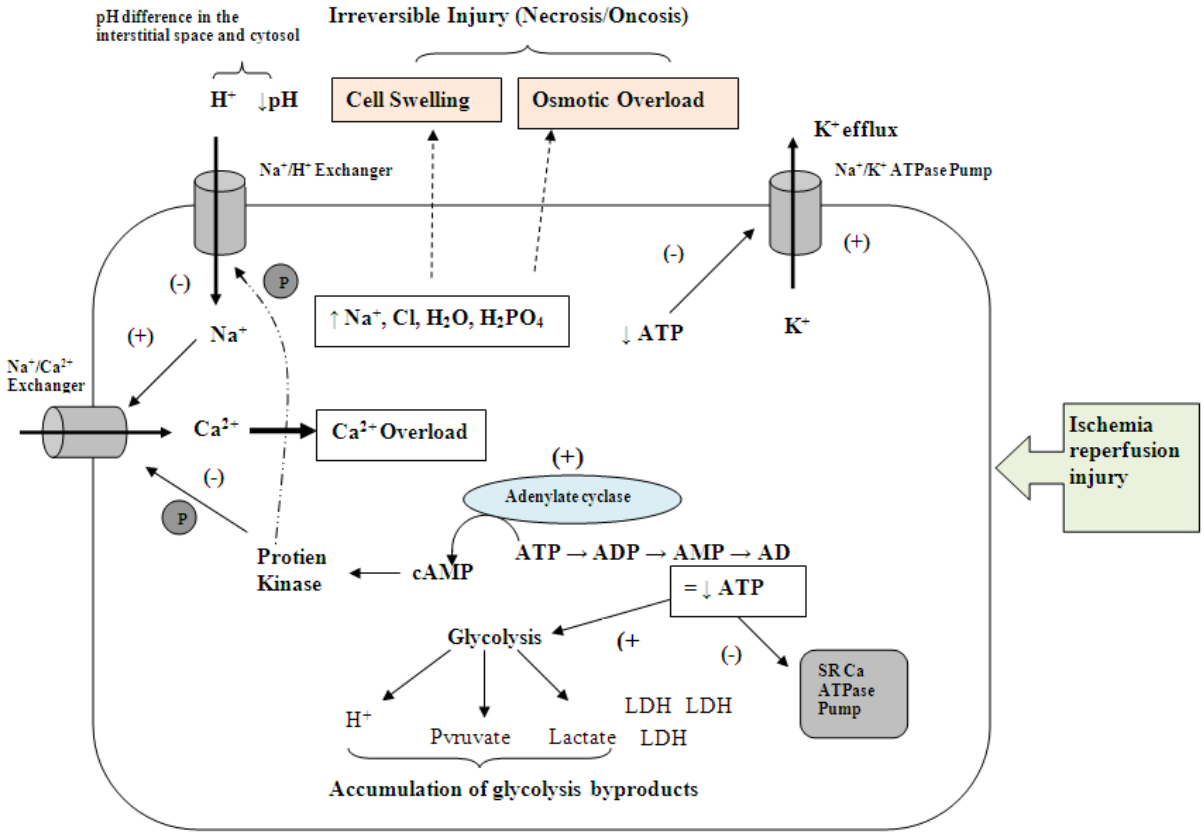


Figure 1.2. Cascade of events during ischemia and reperfusion injury inside the cytosol and interstitial space in cardiomyocytes based on the literature presently available.

(+) = activation, (-) = inhibition

1.2.3. Pathophysiology of myocardial reperfusion injury

Reperfusion injury is a secondary event after ischemia that occurs by the opening of blood flow into the organ. Reperfusion injury is a complex mechanism, involving mechanical, extracellular and intracellular processes (Penna *et al.*, 2008). Reperfusion injury leads to the activation of an inflammatory cascade, functional impairment, apoptosis and necrosis in cardiomyocytes (Buja 2005). The primary mechanism for reperfusion injury has not been fully revealed. However, studies have suggested that overproduction of oxygen free radicals, increased intracellular calcium levels and neutrophil activation during the initial phase of reperfusion are involved. Various other factors are also thought to be involved in reperfusion injury including leukocyte infiltration, platelet and compliment factors and renin-angiotensin system (Buja 2005; Moens *et al.,* 2005).

1.2.3.1. Oxygen free radicals

Reperfusion restores the flow of oxygen to the tissue, generating potent free radicals in humans with ischemic heart disease (Moens *et al.*, 2005). Mitochondrial respiration, xanthine oxidase activity and neutrophil activation are mainly responsible for oxygen free radical production. Higher oxygen free radical concentration leads to lipid peroxidation which diminishes membrane integrity and causes necrosis of cardiomyocytes (Figure 1.3) (Vanden Hoek *et al.,* 1997; Buja 2005; Moens *et al.,* 2005).

Free radicals such as superoxide cause damage to blood vessels by increasing leukocyte adhesion to the walls of blood vessels. Ischemia causes the breakdown of ATP to xanthine. During reperfusion, the endothelial enzyme, xanthine oxidase, converts xanthine and

oxygen to superoxide. Nitric oxide plays a protective role during reperfusion due to its increased production in endothelial cells (Ferdinandy *et al.*, 2003). During reperfusion, abnormally high amounts of superoxide convert almost all the available nitric oxide to peroxynitrite thereby causing damage to the endothelial cells (Ferdinandy *et al.*, 2003). Superoxide dismutase (SOD) converts superoxide radicals to hydrogen peroxide; catalase and glutathione peroxidase (GPX) catalyse the decomposition reaction of hydrogen peroxide to form water and oxygen and thus protect the cell from oxidative damage by H_2O_2 and the hydroxyl radical ($OH^{\bullet}$) (Vanden Hoek *et al.,* 1997; Ceconi *et al.,* 2003).

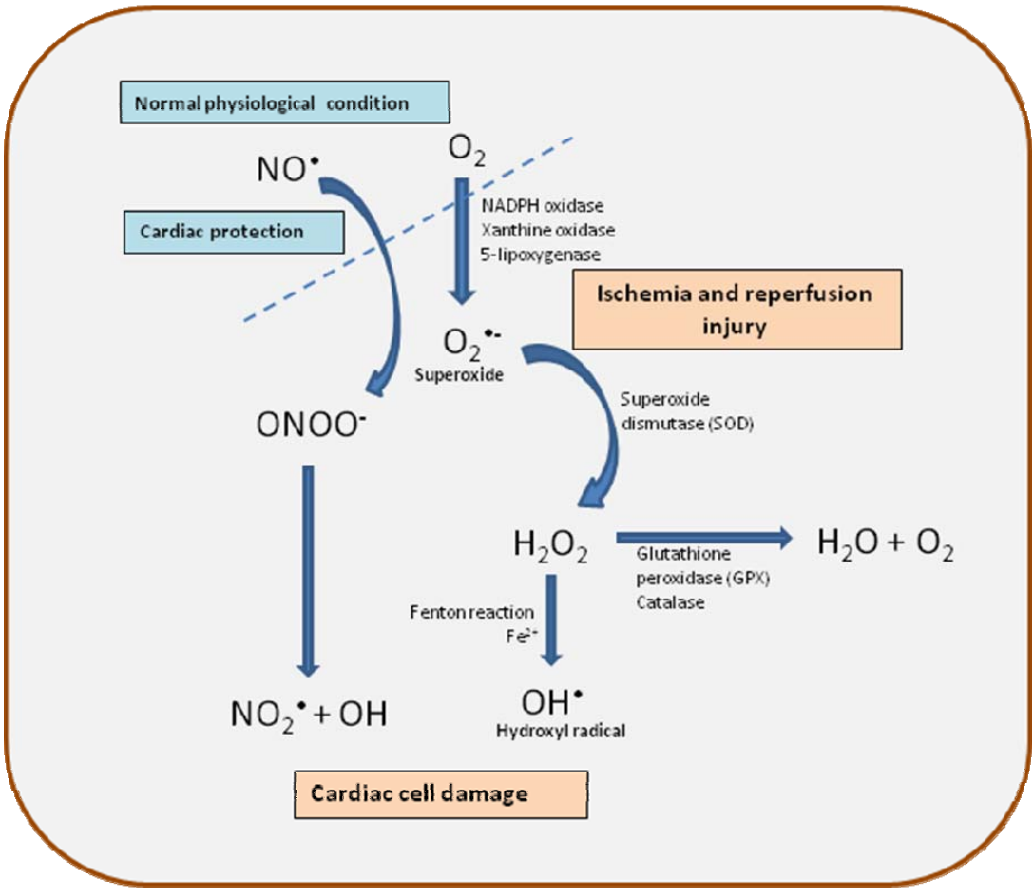


Figure 1.3. ROS production in cardiac cells during ischemia and reperfusion injury.

1.2.3.2. Consequences of ROS production

Reactive oxygen species can attack vital cell components like lipids, proteins and nucleic acids; as well as affecting cellular properties like fluidity, ion transport, enzyme activity, protein synthesis and DNA damage, ultimately resulting in cell death (Becker 2004; Lopez-Neblina *et al.,* 2006).

Lipid peroxidation is initiated by the hydroxyl ($OH^{\bullet}$), alkoxy ($RO^{\bullet}$) and peroxy ($ROO^{\bullet}$) radicals. The presence of a double bond adjacent to the methylene group in polyunsaturated fatty acid makes the methylene C-H bond weaker and therefore hydrogen becomes more prone to abstraction. Lipid peroxidation results in a progressive loss of membrane fluidity, decrease in membrane potential and increase in membrane permeability to certain ions, such as Ca^{2+}, and production of malondialdehyde (MDA) (Ceconi *et al.,* 2003; Becker 2004; Lopez-Neblina *et al.,* 2006)

Protein oxidation results in the loss of critical -SH groups in addition to modifications of amino acids which can lead to formation of carbonyl and other oxidized moieties. Oxidized proteins are much more susceptible to proteolysis and oxidation of proteins can cause damage to ion transport mechanisms, in particular Ca^{2+}, leading to cell injury (Becker 2004; Lopez-Neblina *et al.,* 2006).

Following peroxide mediated DNA damage, the enzyme poly (ADP-ribose) polymerase (PARP) is activated and utilizes large amounts of NAD to repair DNA damage. This

impairs the cell's ability to produce ATP, thus leading to energy deficiency, changes in Ca^{2+} concentration and cell death (Becker 2004; Lopez-Neblina *et al.,* 2006).

1.2.3.3. Calcium overload, hypercontracture and the 'no-flow' phenomenon

During reperfusion the cell accumulates more calcium due to changes in cell homeostasis. In the initial phase of reperfusion, the Na^+/H^+ exchanger and Na^+/HCO_3^- transporters on the plasma membrane are activated because of the decreased pH in the cytosol. The Na^+/H^+ exchanger becomes activated due to the quick influx of Na^+ inside the cytosol, leading to increased Ca^{2+} in the cytosol and Ca^{2+} overload in the cell (Piper *et al.*, 1998; Solaini *et al.*, 2005). Reduced intracellular ATP levels also result in calcium overload in the cytoplasm leading to the inactivation of sacrcoplasmic reticulum Ca^{2+}-ATPase and Na^+/K^+-ATPase (Solaini *et al.*, 2005). After ischemia, excessive cytosolic Ca^{2+} overload was observed in the cardiomyocytes. During initial phase of reperfusion reverse Na^+/Ca^{2+} exchange exacerbate the cytosolic Ca^{2+} overload, which causes the hypercontracture. This increased cytosolic Ca^{2+} increase the accumulation of mitochondrial Ca^{2+}, which has harmful effects on mitochondrial function (Piper *et al.,* 2004; Solaini *et al.,* 2005).

The overload of Ca^{2+} during reperfusion increases cellular osmolarity, which causes swelling of the myocardium cells. Modified cytosolic Ca^{2+} during ischemia increases contractile activation and structural fragility. These effects lead to the further increase of ventricular diastolic pressure as well as necrosis (Piper *et al.,* 2003; Penna *et al.,* 2008). Calcium overload is also thought to be involved in the opening of the mitochondrial permeability transition pore (mPTP). During the reperfusion period, mPTP opening is facilitated by oxidative stress, higher intramitochondrial Ca^{2+} levels and reduced ATP

levels (Moens *et al.*, 2005). However, during the ischemic period acidosis prevents mPTP opening (Penna *et al.*, 2008). Previous studies support the role of both oxygen free radicals and calcium overload in reperfusion injury, as the calcium overload initiates free radical generation by the mitochondria (Ferrari *et al.*, 1993). Reperfusion calcium overload evokes the continuous shortening of cardiac cells causing mechanical rigidity and tissue necrosis, known as hypercontracture of the heart (Moens *et al.*, 2005).

Nuclear factor kappa B (NFκB) activation increases the level of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) genes during ischemia, increasing the availability of nitric oxide. However, during the reperfusion period, NFκB increases inflammatory reactions and worsens myocardial injury (Penna *et al.*, 2008). Nuclear factor kappa B activates the cellular adhesion molecules and increases the adhesion of leukocytes to the endothelium and their movement within the myocardium (Baldwin 2001). Reduced nitric oxide levels cause vasoconstriction and thrombi formation in the coronary vessels. This microvascular dysfunction reduces adequate perfusion after reperfusion and is known as the "no-reflow" phenomenon. It is found to be linked with events like myocardial infarction and cell death (Moens *et al.*, 2005).

1.2.3.4. Other factors

Various other factors such as neutrophil infiltration, platelet infiltration, leukocyte infiltration and renin-angiotensin and complement activation are believed to be involved in reperfusion injury. Neutrophils contribute to reperfusion injury by generating free radicals, proinflammatory mediators, which further increases neutrophil infiltration and causes

myocardial death (Jordan *et al.*, 1999; Moens *et al.*, 2005). The renin-angiotensin system produces angiotensin II, which increases intracellular calcium in cardiac and smooth muscle cells and leads to diastolic dysfunction, coronary vasoconstriction and increased cardiac contraction. These events cause cardiac cell death during reperfusion injury. Reperfusion injury also causes platelet aggregation, a consequence of which can be coronary blockage (Moens *et al.*, 2005).

Myocardial ischemia-reperfusion injury can provoke apoptosis, oncosis and necrosis. Apoptosis and oncosis refer to programmed cell death, which describes the cellular structural changes and damage before cell death. However, necrosis is the end stage of the cell death process (Penna *et al.*, 2008). Principal cellular and molecular events involved in ischemia-reperfusion injury and myocardial protection are shown in Figure 1.4.

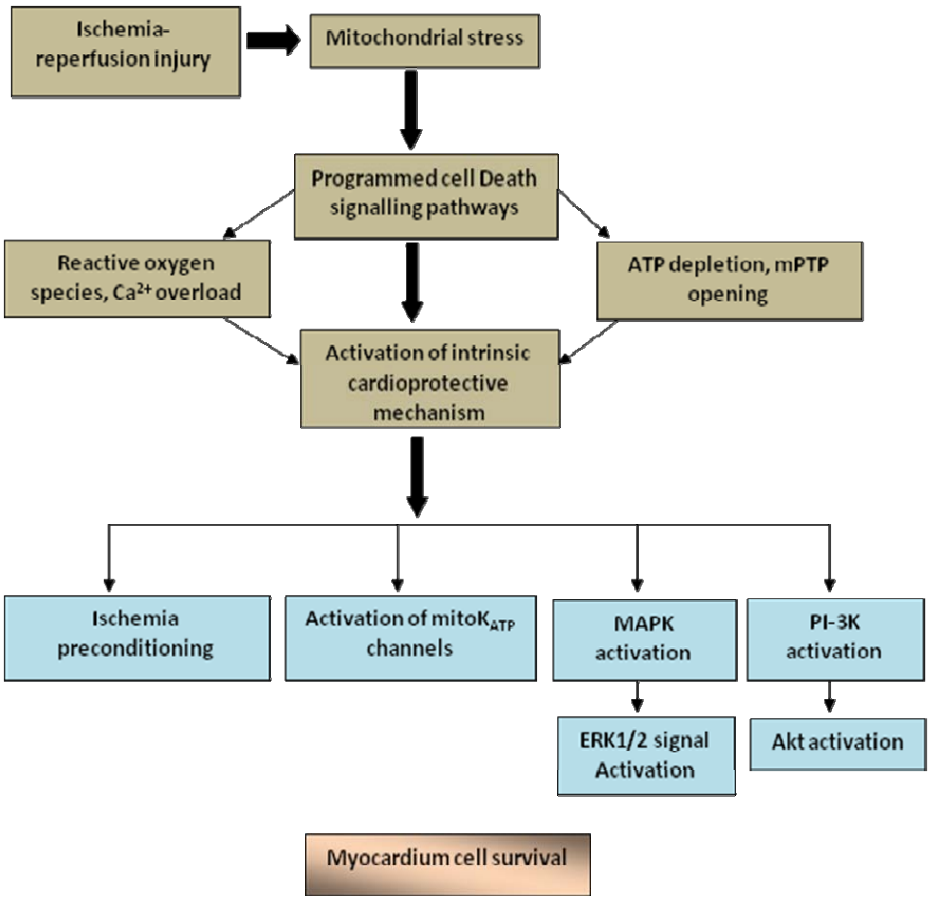


Figure 1.4. Cellular mechanisms involved in protection against programmed cell death based on the literature currently available.

1.2.4 Adenosine and ischemia-reperfusion injury

Pathophysiological conditions, such as ischemia and ischemia-reperfusion injury, can release adenosine as an endogenous cardioprotectant. Adenosine is a ubiquitous multipurpose signal molecule found in various locations in the body under normal physiological conditions as well as during pathophysiological conditions, and activates G protein coupled receptors. Activation of adenosine receptors mediates cardioprotection through various signalling pathways, which is described in further detail in the following section.

1.3. Adenosine

The purine nucleoside, adenosine, is a naturally occurring, physiologically active molecule that is produced in many sites of the body in response to multiple types of stimuli such as hypoxia, ischemia-reperfusion injury and high or low metabolic activity (Berne 1963; Hori *et al.,* 1991; Ely *et al.,* 1992a; Ralevic *et al.,* 1998; Jacobson *et al.,* 2006). Adenosine acts as a regulatory substance and plays an important role in coronary circulation, as well as metabolic regulation of the nervous and endocrine systems (Donato *et al.*, 2003). Adenosine exerts a wide range of physiological effects including negative inotropic and chronotropic effects, vasodilation, neutralization of adrenergic stimulation and inhibition of inflammatory responses in the heart, as well as neuromodulatory effects such as a decrease in electrical excitability and inhibition of excitatory amino acid release in the brain (Table 1.5) (Engler 1991; Hori *et al.,* 1991). Due to decreased oxygen supply under hypoxic and ischemia-reperfusion conditions, increased adenosine production takes place by the metabolic conversion of ATP to AMP and further to adenosine, supporting the statement that, "Adenosine is indeed the indicator of cell viability, the signal of life" (Engler 1991).

1.3.1. Source of extracellular adenosine

Adenosine is present as a purine nucleoside inside tissues, generated by the metabolism of ATP, and is transported into the extracellular environment by nucleoside transporters (Thorn *et al.,* 1996; Godecke 2008). Extracellular adenosine is synthesized by the AMP-adenosine pathway and adenosine uptake mechanism, which is described in a subsequent section. Another source of intracellular adenosine is S-adenylhomocysteine (SAH), generated from S-adenosylmethionine. The enzyme SAH hydroxylase reversibly catalyses

SAH to form intracellular homocysteine and adenosine and also binds with adenosine (Belardinelli *et al.*, 1989). S-adenylhomocysteine-generated adenosine is slowly released into the cytosol as part of the de-novo synthesis pathway of adenosine (Figure 1.5).

1.3.1.1. The extracellular cAMP-adenosine pathway

Both intracellular and extracellular adenosine is derived from ATP through the conversion of ADP and AMP (Jackson *et al.,* 2007; Godecke 2008). The intracellular formation of adenosine is dependent on the activity of a cytosolic enzyme called 5'-nucleotidase. Subsequently adenosine is released into the extracellular compartment by the adenosine transporter (Godecke 2008). In the extracellular environment an enzyme called ecto-nucleoside triphosphate diphosphohydrolase (eNTPD or CD39) catalyses ATP breakdown to AMP. In the interstitial compartment ecto-phosphodiesterase (e-PDE) and ecto-5'-nucleotidase (e-5'-NTase or CD73) converts extracellular AMP to adenosine (Jackson *et al.*, 2007), which activates adenosine receptors (Figure 1.5).

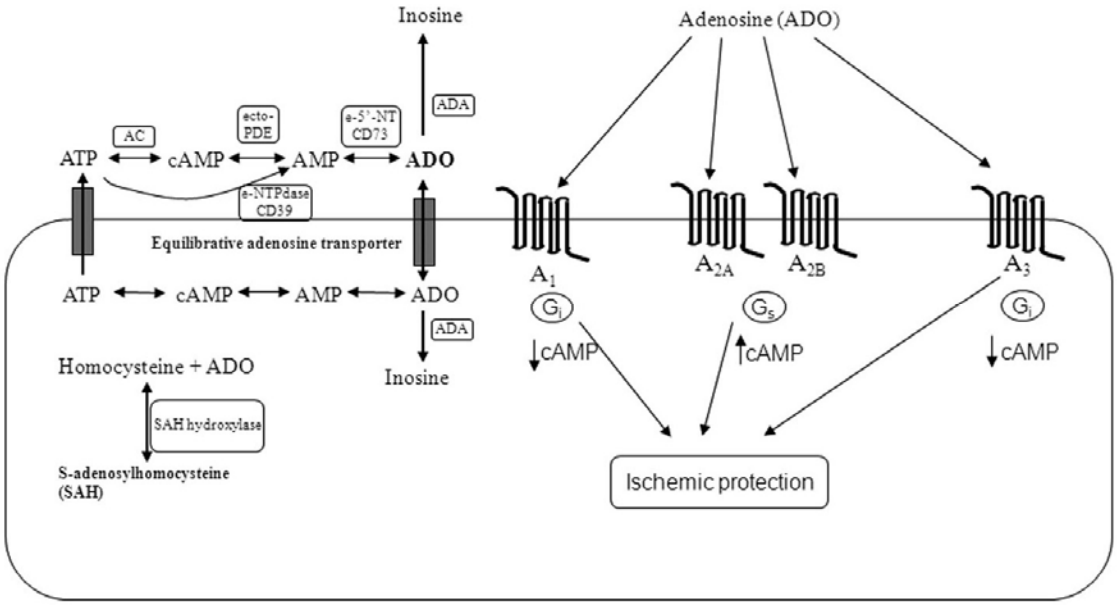


Figure 1.5. Source of extracellular adenosine by the metabolic degradation of ATP and intracellular adenosine release based on the literature currently available.

1.3.2. Adenosine uptake and metabolism

Extracellular adenosine is metabolised very quickly, therefore its level also drops rapidly (Noji *et al.*, 2004). Adenosine in the blood has a very short half life (30 s) and is metabolised in less than one minute (Belardinelli *et al.*, 1989; Shryock *et al.*, 1997). Adenosine is recycled or catabolised outside the cell and taken up by adenosine transporters. During transport adenosine mainly competes with adenosine deaminase or adenosine kinase, which converts adenosine to inosine or AMP (Shryock *et al.*, 1997). Adenosine transport by the nucleoside transporter plays a key role in maintenance of extracellular adenosine concentration (Noji *et al.*, 2004). Nucleoside transporters in the cardiac tissue exist in two forms, equilibrative (passive, facilitated diffusion) and concentrative (active, sodium ion-dependent). Equilibrative transporters transport adenosine in either direction depending on the concentration gradient (Figure 1.5).

Concentrative transport uses energy from the sodium ion gradient across the plasma membrane to promote the influx of adenosine against the concentration gradient (Griffith *et al.*, 1996). To enhance the pathophysiological response of adenosine, adenosine uptake plays a key role in maintaining the extracellular adenosine concentration (Noji *et al.*, 2004).

1.3.3. Adenosine receptor classification and structure activity relationship (SAR)

1.3.3.1. Adenosine and its SAR

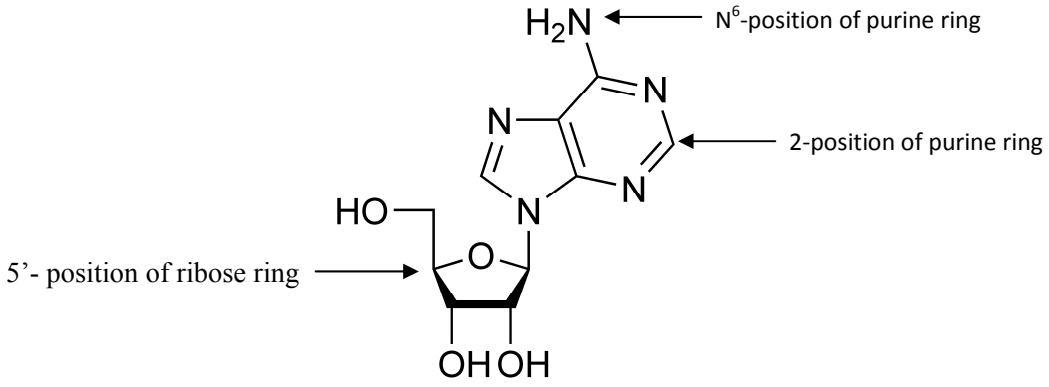


Figure 1.6. Chemical structure of adenosine.

Adenosine is a multipurpose signalling molecule that regulates a variety of cellular functions and is released under conditions of physiological stress. The chemical structure of adenosine is shown in Figure 1.6. Adenosine consists of a ribose sugar ring attached to a purine-furan complex via a β-N9-glycosidic bond. Adenosine acts through four subtypes of receptors (adenosine receptors), and the receptor subtype affinity of adenosine can be modified by attachment of various chemical moieties at the 5' and 2' position of the purine ring and 5' position of the ribose ring. The four adenosine receptor subtypes are A_1, A_{2A},

A_{2B} and A_3; all of which have been well described in the literature (Ralevic *et al.,* 1998; Fredholm *et al.,* 2001; Linden 2001; Burnstock 2002; Jacobson *et al.,* 2006). Adenosine provides a first line defence against pathophysiological conditions like ischemia and reperfusion injury by increasing the ratio of oxygen supply and demand, cell conditioning, triggering an anti-inflammatory response and promotion of angiogenesis (Hori *et al.,* 1991; Donato *et al.,* 2003; Jacobson *et al.,* 2006; Tikh *et al.,* 2006). Modification at the N^6-position shows the adenosine A_1 receptor selective adenosine derivatives. The oxidation of the 5'-carbon of the ribose, amidation of the resulting uronic acid and *N*-substitution of the amide is tolerated in the ribose moiety of adenosine. Removal of the 2'- and 3'- hydroxyl groups completely abolishes agonistic activity of the adenosine A_1 receptor agonist N^6-cyclohexyladenosine. Modification at the 5'- position with carboxyethylamide and substitution with ethylamide at 2'-position of purine increases the affinity for the adenosine A_{2A} receptor, which lead to the development of the NECA derivative CGS 21680 (2-[*p*-(2-carboxyethyl)phenethylamino] adenosine-5'-*N*-ethyluronamide. The introduction of N^6-substituents into 5'- modified adenosine derivatives have shown a high affinity for the adenosine A_3 receptor. The 5'- modification in the 5'-*N*-methyluronamide is resulted in the highest potency for the adenosine A_3 receptor. N^6-benzyl-5'-*N*-methyl-carboxamideadenosine (MECA) have shown high affinity for the rat adenosine A_3 receptor. Modification at the 5'-position as uronamide and/ or as N^6-benzyl derivatives increases the affinity for the adenosine A_3 receptor. Substitution of the benzyl group with nitro and halo groups at the 3'- position mainly enhances the affinity and selectivity at the adenosine A_3 receptor.

1.3.3.2. Adenosine receptor classification

Adenosine receptors, also known as P1 receptors, are G protein coupled receptors (GPCRs). Adenosine receptors have been classified into four subtypes: A_1, A_{2A}, A_{2B} and A_3 (Fredholm *et al.*, 1994; Fredholm *et al.*, 2001). The adenosine A_1 and A_3 receptors both couple to the inhibitory $G_{i/o}$ protein, which inhibits adenylate cyclase, while the high affinity adenosine A_{2A} and low affinity A_{2B} receptors both couple to the stimulatory G_s protein (Belardinelli *et al.,* 1989; Hori *et al.,* 1991; Ely *et al.,* 1992a; Ralevic *et al.,* 1998; Auchampach *et al.,* 1999; Fredholm *et al.,* 2001; Linden 2001; Jacobson *et al.,* 2006). The adenosine A_{2B} receptor can also couple to the $G_{q/11}$ protein to mediate its effect on calcium mobilization (Linden 2001; Jacobson *et al.,* 2006).

1.3.3.3. Pharmacological classification of adenosine receptors

Prior to the full characterization of adenosine receptors using cloning techniques, a range of agonist and antagonist binding properties, and agonist potencies were used to classify adenosine receptors (Fredholm *et al.*, 2001). The selective agonists and antagonists remain important tools for characterization and functional determination of adenosine receptors. Different adenosine receptor agonists and antagonists and their binding efficacy to human adenosine A_1, A_{2A}, A_{2B} and A_3 receptors are shown in Tables 1.1 and 1.2. These pharmacologically active ligands have specific potency and selectivity towards the adenosine receptor subtypes (Figure 1.7, 1.8).

Table 1.1. Binding efficacy of various pharmacological adenosine receptor agonists, in decreasing order of agonist potency towards adenosine receptor subtypes.

For adenosine A_1 receptor; Reference: (Fredholm *et al.*, 2001; Jacobson *et al.*, 2006): RPIA (1 nM) > NECA (6 nM) >IB-MECA (54 nM) > CGS21680 (289 nM)
For adenosine A_{2A} receptor; Reference: (Fredholm *et al.*, 2001; Jacobson *et al.*, 2006): NECA (10 nM) = CGS21680 (15 nM) > IB-MECA (56 nM) > RPIA (124 nM)
For A_{2B} adenosine receptor; Reference: (Fredholm *et al.*, 2001; Jacobson *et al.*, 2006): NECA (2000 nM) > RPIA (> 10,000 nM) =IB-MECA (>10,000 nM) > CGS21680 (>10,000 nM)
For A_3 adenosine receptor; Reference: (Fredholm *et al.*, 2001; Jacobson *et al.*, 2006): IB-MECA (1 nM) > NECA (113 nM) = RPIA (158 nM) > CGS21680 (584 nM)

Table 1.2 (A, B) shows the selective adenosine agonists and antagonists for different adenosine receptor subtypes as pharmacological tools for receptor classification. Novel selective adenosine agonists (VCP series) were synthesized by the Department of Medicinal Chemistry (Monash University, Parkville, VIC, Australia).

1.3.3.3.1. Adenosine A_1 receptor pharmacology

For pharmacological studies in human, rat and mouse, the selective adenosine A_1 receptor agonists CCPA, CPA and antagonist DPCPX are routinely used (Fredholm *et al.*, 2001). DPCPX (A_1 antagonist) was used to confirm the adenosine A_1 receptor-mediated effect in post-ischemic contractile recovery in a cardiomyocyte simulated ischemia model (Stambaugh *et al.*, 1997), and isolated mouse heart ischemia reperfusion model (Peart *et al.,* 2000; Butcher *et al.,* 2007) DPCPX (K_i 3.9 nM for adenosine A_1 receptor). It has been reported that DPCPX also interacts with the adenosine A_{2B} receptor to some extent (Fredholm *et al.*, 2001), but this may not be an issue as reports from an A_1 knockout mouse have shown a lack of binding for DPCPX (Johansson *et al.*, 2001). Another agonist with high affinity and selectivity for the adenosine A_1 receptor is R-N^6-(methyl-2-phenylethyl)adenosine (RPIA, K_i 1 nM for adenosine A_1 receptor). Tables 1.1 and 1.2 (A, B) shows agonists available as pharmacological tools.

The result of the present study should be interpreted with a caveat that the adenosine A_1 and A_{2A} antagonist DPCPX and ZM241385 respectively, have a relatively high binding affinity for the adenosine A_{2B} receptor as well in various rodent species, which has been reported recently (Auchampach *et al.,* 2009). The adenosine A_{2B} antagonist MRS1754 (200 nM)

used in the present study has shown some binding affinity for the adenosine A_1 receptor in rat (Table 1.2 B). In hindsight, a lower concentration of DPCPX and MRS1754 would have been appropriate.

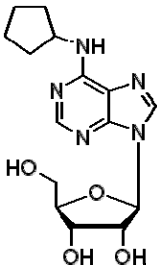
Table 1.2 (A). Binding affinity of agonists and antagonists to human adenosine receptor subtypes used in the present study (Fredholm *et al.,* 2001; Jacobson *et al.,* 2006; Gregg *et al.,* 2007; Devine *et al.,* 2010), [#]our unpublished data

Adenosine Receptor (AR) Subtypes		Ki value for AR (nM)			
		Hu A_1AR	**Hu A_{2A}AR**	**Hu A_{2B}AR**	**Hu A_3AR**
Tools agonist	**A_1 CPA** (N^6-cyclopentyl adenosine)	2.3	794	18,600	72
	A_{2A} CGS21680 (4-[2-[[6-Amino-9-(*N*-ethyl-b-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl]benzenepropanoic acid hydrochloride)	289	27	>10,000	67
	A_{2B} LUF5834 (2-amino-4-(4-hydroxyphenyl)-6-(1H-imidazol-2-ylmethylsulfanyl)pyridine-3,5-dicarbonitrile)	2.6	28	12	538
	A_1/ A_2 NECA (5'-*N*-Ethylcarboxamidoadenosine 1-(6-Amino-9H-purin-9-yl)-1-deoxy-*N*-ethyl-b-D-ribofuran uronamide)	14	20	140	25
	A_3 IB-MECA (N^6-(3-iodobenzyl)-adenosine-5'-*N*-methylcarboxamide)	51	2,900	11,000	1.8
Novel agonists	**A_1 VCP28** (N^6-(2,2,5,5-tetramethylpyrrolidin-1-yloxyl-3-ylmethyl)adenosine)	50	>10,000	>10,000	21,200
	A_3 VCP103[#] (*N*-Methyl-N^6-[4-[2-[3,5-di-tert-butyl-4-hydroxybenzamido]ethyl]phenyl]adenosine-5′-uronamide)	243	>10,000	6960	45

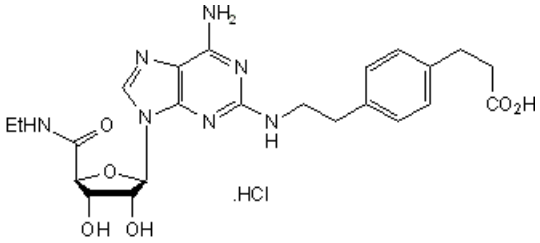
	A_3 VCP438# (*N*-Methyl-N^6-[4-[2-[3,5-di-*tert*-butylbenzamido]ethyl]phenyl]adenosin-5′-uronamide)	245	>10,000	Not disclosed	2.25
	A_3 VCP439# (*N*-Methyl-N^6-[4-[2-[3,4,5-trimethoxybenzamido]ethyl]phenyl]adenosin-5′-uronamide)	26.2	27% at 10 μM	Not disclosed	4.26
	A_3 VCP485# (*N*-Methyl-N^6-[4-[2-[3,4,5-trimethoxybenzamido]ethyl]benzyl]adenosin-5′-uronamide)	76.8	>10,000	Not disclosed	5.04
	A_3 VCP486# (*N*-Methyl-N^6-[4-[2-[3,5-di-*tert*-butyl-4-hydroxybenzamido]ethyl]benzyl]adenosin-5′-uronamide)	105	>10,000	Not disclosed	5.63
	A_3 VCP487# (*N*-Methyl-N^6-[4-[2-[3,5-di-*tert*-butylbenzamido]ethyl]benzyl]adenosin-5′-uronamide)	128	>10,000	Not disclosed	9.02
Tools antagonist	**A_1 DPCPX** (8-cyclopentyl-1,3-dipropylxanthine)	3.9	129	56	3,980
	A_{2A} ZM241385 (4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]t riazin-5-ylamino]ethyl)phenol)	774	1.6	75	743
	A_{2B} MRS1754 (*N*-(4-Cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]-acetamide)	403	503	2	570
	A_3 MRS1191 (3-Ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate)	Not disclosed	Not disclosed	Not disclosed	31

Table: 1.2 (B). Binding affinities of agonists and antagonists to rat (Rt), mouse (M), rabbit (R) and dog (D) adenosine receptor (AR) subtypes used in the present study (Jiang *et al.,* 1997; Fredholm *et al.,* 2001; Auchampach *et al.,* 2009).

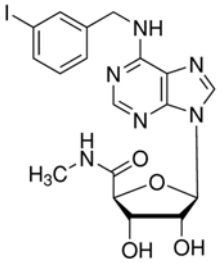
Adenosine Receptor (AR) Subtypes		K_i value for AR (nM)			
		A_1AR	**A_{2A}AR**	**A_{2B}AR**	**A_3AR**
Tools agonist	**A_1 CPA**	0.59^{Rt}	460^{Rt}	Not available (NA)	920^{Rt}
	A_{2A} CGS21680	3100^{Rt}	22^{Rt}	NA	580^{Rt}
	A_1/ A_2 NECA	6.3^{Rt}	10^{Rt}	1001^{M}; 2060^{R}; 3001^{D}	NA
	A_3 IB-MECA	54^{Rt}	56^{Rt}	1204^{M}; 2505^{R}; 2606^{D}	1.1^{Rt}
Tools antagonist	**A_1 DPCPX**	0.3^{Rt}	340^{Rt}	186^{Rt}; 86.2^{M}; 96^{R}; 147^{D}	$>10{,}000^{Rt}$
	A_{2A} ZM241385	$2{,}000^{Rt}$	0.30^{Rt}	31.1^{M}; 109^{R}; 76.2^{D}	$150{,}000^{Rt}$
	A_{2B} MRS1754	17^{Rt}	610^{Rt}	12.8^{Rt}; 3.39^{M}; 1.79^{R}; 12.8^{D}	$>10{,}000^{Rt}$
	A_3 MRS1191	$40{,}000^{Rt}$	$>10{,}000^{Rt}$	NA	31.4^{Rt}



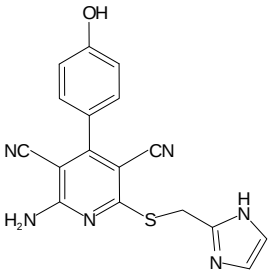
CPA (A_1 selective)



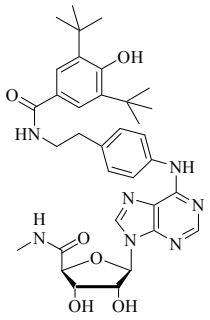
CGS21680 (A_{2A} selective)



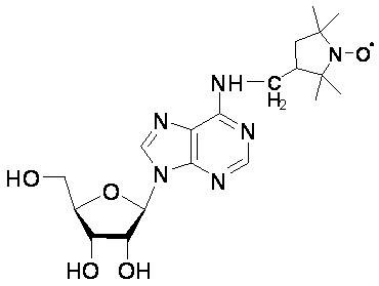
IB-MECA (A_3 selective)



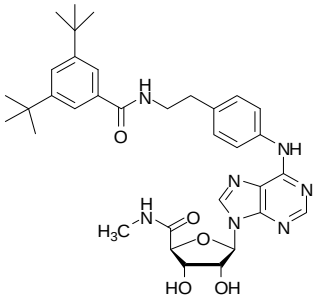
LUF (A_{2B} selective)



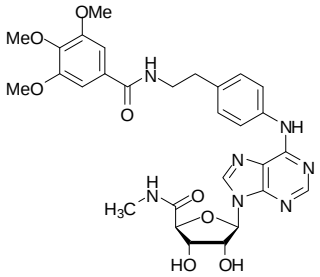
VCP103 (A_3 selective)



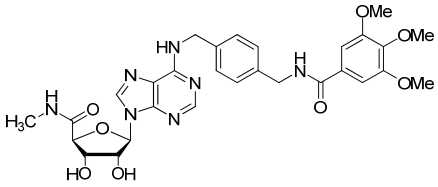
VCP28 (A_1 selective)



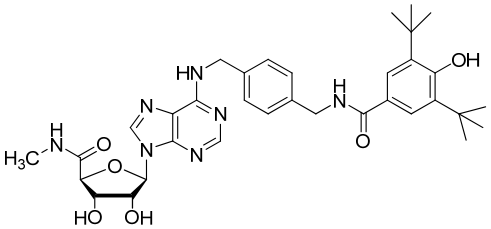
VCP438 (A_3 selective)



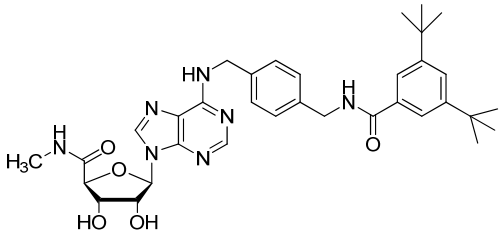
VCP439 (A_3 selective)



VCP485 (A_3 selective)



VCP486 (A_3 selective)



VCP487 (A_3 selective)

Figure 1.7. Chemical structures of some selective adenosine receptor agonists.

1.3.3.3.2. Adenosine A_{2A} receptor pharmacology

The most commonly used A_{2A} selective agonists are CGS21680, DMPA (N^6-[2-(3,5-Dimethoxyphenyl)-2-(2-methylphesnyl)-ethyl]adenosine), HE-NECA (2-(1-Hexyn-1-yl)adenosine-5′-N-ethyluronamide, 2-Hexynyl-5′-ethylcarboxamidoadenosine), ATL-146e and CVT3146. Commonly employed antagonists are ZM241385, KW6002 (Istradefylline), CSC (1,3,7-Trimethyl-8-(3-chlorostyryl)xanthine) and SCH58261 (7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine). A non-selective adenosine agonist, NECA, binds to both the adenosine A_1 and A_2 receptors. An adenosine A_{2A} agonist, CGS21680, with greater affinity has been developed by modification of the NECA structure (Hutchison *et al.,* 1989; Feoktistov *et al.,* 1997). Another selective adenosine A_{2A} agonist, ATL146e, has been developed, which has higher affinity for the human adenosine A_{2A} receptor compared to CGS21680 (Rieger *et al.*, 2001). Various selective antagonists for the adenosine A_{2A} receptor are available. Among them, SCH58261 and ZM241385 are highly selective for the adenosine A_{2A} receptor (K_i 0.6 nM and 1.4 nM respectively) (Dionisotti *et al.*, 1997; Ongini *et al.*, 1999). Endogenous adenosine and exogenous agonist CGS21680 causes coronary vasodilation by activation of adenosine A_{2A} receptor, the effect was antagonised by A_{2A} antagonist SCH58261 in a dose dependent manner using guinea pig isolated perfused heart (Belardinelli *et al.*, 1998). Adenosine A_{2A} receptor deletion and antagonism reduces ROS production and increases the phosphorylation of ERK1/2, p38MAPkinase and JNK by regulation of NADPH oxidase activity in cardiac cells (Ribé *et al.*, 2008). In CHO and HEK293 cells transfected with human adenosine A_{2A} receptors, SCH58261 was shown to be a selective A_{2A} antagonist using radiolabelled ligand binding assays (Dionisotti *et al.*, 1997; Ongini *et al.*, 1999).

Adenosine A_{2A} receptor-mediated post-conditioning cardioprotection was blocked by ZM241385 in an isolated mouse ischemia reperfusion model (Morrison *et al.*, 2007). The adenosine A_{2A} receptor selective antagonist ZM241385 blocked A_{2A} receptor-mediated coronary vasodilation and adenosine A_{2A} receptor selective agonist CGS21680 attenuated ischemia and reperfusion induced stunning in a global ischemia of guinea pig isolated working heart model (Maddock *et al.*, 2001).

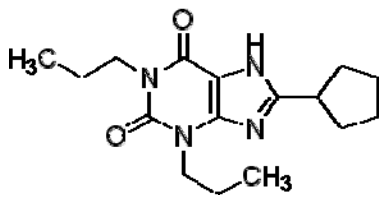
1.3.3.3.3. Adenosine A_{2B} receptor pharmacology

Selective adenosine A_{2B} receptor agonists have been recently developed. LUF5834 and LUF5835 have exhibited high affinity for human adenosine A_{2B} receptors (K_i = 12 nM and 10 nM, respectively) compared to NECA (K_i = 140 nM) (Linden 2001; Beukers *et al.,* 2004; Jacobson *et al.,* 2006). Some potent and highly selective antagonists for the adenosine A_{2B} receptor include MRS1754 and MRS1706 (K_i = 1.97 nM and 1.39 nM, respectively), and are readily available for pharmacological evaluation of adenosine A_{2B} receptors (Ji *et al.,* 2001; Linden 2001).

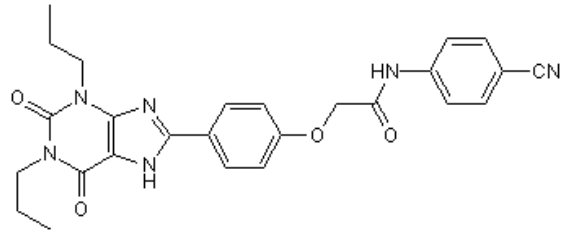
1.3.3.3.4. Adenosine A_3 receptor pharmacology

A number of adenosine A_3 receptor agonists have also been developed recently. IB-MECA, Cl-IB-MECA and APNEA are potent adenosine A_3 receptor agonists with K_i values of 1.8, 0.33 and 15.5 nM, respectively. Because of the adenosine A_3 receptor's insensitive nature towards xanthines (Fredholm *et al.*, 2001), most antagonists for the A_3 receptor have a nonxanthine structure. Isoquinolone, quinazoline, flavanoid and dihydropyridine derivatives are highly selective adenosine A_3 antagonists. MRS1191 (K_i = 31 nM for

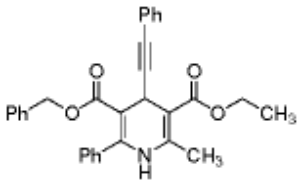
adenosine A_3 receptor), MRS1523, MRS1220 (K_i = 0.7 nM for adenosine A_3 receptor), VUF5574 (K_i = 4 nM for adenosine A_3 receptor) and MRS1292 are the selective adenosine A_3 antagonists which are currently available. Adenosine A_3 receptor-mediated ischemic protection is blocked by a selective A_3 antagonist, MRS1191, in a cultured chicken ventricle myocyte ischemia model (Liang *et al.*, 1998). One study reported that IB-MECA-mediated reduction in necrosis and improved post-ischemic recovery was abolished by MRS1191 in a Langendorff perfused isolated mouse heart ischemia reperfusion model (Zhao *et al.*, 2002).



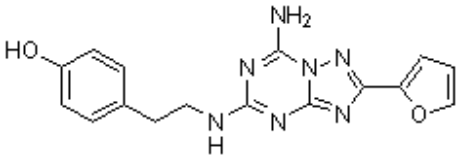
DPCPX (A_1 antagonist)



MRS1754 (A_{2B} antagonist)



MRS1191 (A_3 antagonist)



ZM241385 (A_{2A} antagonist)

Figure 1.8. Chemical structures of some selective adenosine receptor antagonists.

1.4. Adenosine receptor subtype expression

1.4.1. Organ and cellular distribution of adenosine receptors

Adenosine receptors are widely distributed in different tissues of the body. Cardiac and general tissue distribution of the adenosine receptors are described in Table 1.3.

Table 1.3. Distribution of adenosine receptors (Feoktistov *et al.,* 1997; Sommerschild *et al.,* 2000; Fredholm *et al.,* 2001)

A_1	A_{2A}	A_{2B}	A_3
Cardiac- Atrial myocytes, ventricular myocytes, AV node, SA node **Other**- brain region (cerebral cortex, hippocampus, brain stem, thalamus, spinal cord), liver, kidney, lungs, small intestine, spleen, eye, stomach, reproductive system (vas deference, testes)	**Cardiac**- Coronary endothelium (guinea pig, mouse), coronary VSMC (guinea pig, mouse), ventricular myocytes, mast cell, Monocytes, Macrophages, Eosinophils), blood vessels, blood platelets **Other**- brain region (GABAergic neurons), lungs, immune cells, spleen, thymus leukocytes (lymphocytes and granulocytes)	**Cardiac-** Coronary endothelium (rat), coronary VSMC (rat), blood vessels **Other**- Colon, bladder, cecum, lungs, eye, mast cells, liver, kidney, ovary, adipose tissue, adrenal gland, pituitary gland, brian	**Cardiac**- Coronary VSMC (rat), ventricular myocytes, Mast cells **Other**- mast cells (rat), testes (rat, human), thyroid, brain, kidney, intestine, eye, spleen, liver, adrenal gland

1.4.2. Adenosine receptor structure and signalling

Adenosine acts on receptor subtypes A_1, A_{2A}, A_{2B}, and A_3. These receptors belong to the G-protein-coupled receptors (GPCR) family, and are widely distributed on the cell surface (Palmer *et al.*, 1995; Fredholm *et al.*, 2000; Fredholm *et al.*, 2001; Linden 2001). Adenosine receptors typically use cyclic adenosine monophosphate (cAMP) as a second messenger (Hasko *et al.*, 2008). Adenosine A_1 and A_3 receptors act through G_i proteins and inhibit adenylate cyclase activity, leading to a decrease in the cAMP level inside the cells (Fredholm 2007). However, adenosine A_{2A} and A_{2B} receptors act through the G_s protein and stimulate adenylate cyclase, increasing cAMP levels in the cell (Figure 1.9) (Fredholm 2007).

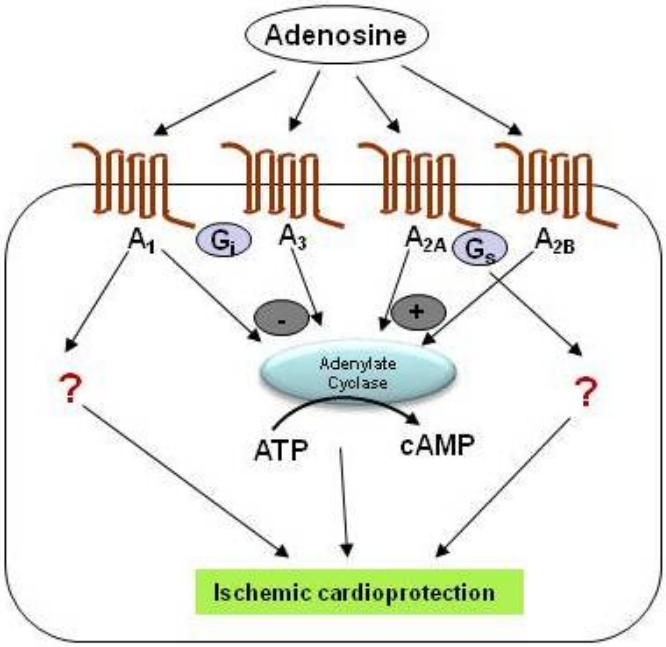


Figure 1.9. Endogenous adenosine signalling pathway.

Adenosine receptor activation involves cAMP-independent signalling pathways, which may be involved in cardioprotection. Increased level of adenosine during ischemic conditions activates various signalling pathways, including PKC, PKA, PKG, Akt and ERK1/2 (Schulte *et al.,* 2000; Germack *et al.,* 2004; Kuno *et al.,* 2007; Kuno *et al.,* 2008). Activation of the adenosine A_1 and A_3 receptors phosphorylates the Na^+/H^+ exchanger, reducing Na^+ overload inside cardiomyocytes and providing cardioprotection (see Figure 1.2). Endogenous adenosine released during ischemic conditions has antiadrenergic effects, which diminishes the levels of norepinephrine, reducing the inotropic and metabolic effects on the ischemic heart. Adenosine binding on blood vessels results in vasodilation effects, which reduces reperfusion injury, myocardial stunning and arrhythmia (Sommerschild *et al.*, 2000).

1.4.3. Adenosine receptor signal transduction pathways and cardioprotection

Intracellular signalling pathways recruited by adenosine receptors depend on the coupling to the effectors protein via G protein subunits. The adenosine receptor signal transduction mechanism involves G protein coupling, second messenger effects and downstream signalling pathways (Table 1.4).

1.4.4. G protein coupling to second messengers

Originally adenosine receptors were classified on the basis of their effect on adenylate cyclase. Adenosine A_1 and A_3 receptors are coupled to the $G_{i/0}$ protein which inhibits adenylate cyclase resulting in reduced cAMP levels (Linden 2001; Mubagwa *et al.,* 2001). Adenosine A_1 receptor activation increases the level of inositol-1-4-5-triphosphate (IP_3) and

diacylglycerol (DAG) through its effect on phospholipase C (PLC) under pathophysiological conditions such as ischemia and reperfusion injury. Ischemia and reperfusion stimulates intracellular calcium release from the endoplasmic reticulum and sarcoplasmic reticulum, and influx of extracellular calcium through the calcium entry pathways (Dickenson *et al.*, 1993), subsequently activating protein kinase C (PKC), phospholipase A_2 (PLA_2), nitric oxide synthase (NO) and Ca^{2+}-dependent K channels (Olah *et al.*, 1995; Ralevic *et al.*, 1998). The adenosine A_3 receptor is also coupled to the $G_{q/11}$ protein which increases the signalling of IP_3, DAG and PLC (Palmer *et al.,* 1995; Ralevic *et al.,* 1998; Linden 2001). Conversely, adenosine A_{2A} and A_{2B} receptors are coupled to the G_s protein, which activates adenylate cyclase and increases the level of cAMP. These receptors are also coupled to $G_{q/11}$ protein, which activates IP_3/DAG and PLC signalling pathways (Olah *et al.,* 1995; Gao *et al.,* 1999; Linden *et al.,* 1999).

Table 1.4. Adenosine receptor G protein coupling and signalling transduction mechanisms.

Adenosine receptor subtype	Coupling G protein	Effect on adenylate cyclase (AC)	Signal transduction mechanism/effect of G protein coupling	References
A_1	G_{i1-3} G_0	Inhibition	↓cAMP, ↑ PLC, ↑ PKC, ↑ K_{ATP}	(Shryock *et al.,* 1997; Sommerschild *et al.,* 2000; Fredholm *et al.,* 2001; Mubagwa *et al.,* 2001; Jacobson *et al.,* 2006)
A_{2A}	G_s	Stimulation	↑cAMP, ↑IP_3, ↑K_{ATP}	(Olah *et al.,* 1995; Palmer *et al.,* 1995; Fredholm *et al.,* 2001; Linden 2001; Jacobson *et al.,* 2006)
A_{2B}	G_s $G_{q/11}$	Stimulation	↑cAMP ↑IP_3, ↑PLC	(Gao *et al.,* 1999; Linden *et al.,* 1999; Fredholm *et al.,* 2001; Linden 2001; Jacobson *et al.,* 2006)
A_3	G_{i2-3} $G_{q/11}$	Inhibition	↓cAMP ↑ IP_3,↑ PLC	(Palmer *et al.,* 1995; Fredholm *et al.,* 2001; Jacobson *et al.,* 2006)

1.5. Adenosine receptor activation and resultant physiological effects on various organ systems

During various pathophysiological conditions such as hypoxia and ischemia-reperfusion injury, adenosine receptor activation either decreases oxygen demand by reduced work load through activation of the A_1 adenosine receptor, or increases oxygen supply to the tissue via vasodilation effects through activation of adenosine A_{2A} and A_{2B} receptors. Adenosine released during pathophysiological conditions modulates a variety of physiological effects by acting on different adenosine receptor subtypes depending on the location and distribution of the receptor subtypes. Adenosine receptor activation has distinct effects on most organ systems, including the cardiovascular, central nervous, respiratory, immune, renal, digestive and reproductive systems, as summarised in Table 1.5.

Adenosine receptors mediate a variety of cardioprotective effects in the cardiovascular system, both cardiac and vascular. Adenosine regulates coronary circulation in the heart (Berne 1980) and also depresses sinoatrial (SA) and atrioventricular (AV) node activity, reduces atrial contractibility, attenuates the contractile response to catecholamines, mainly in the ventricles, and depresses of cardiac automaticity (Belardinelli *et al.*, 1989).

During ischemia-reperfusion injury, adenosine exerts cardioprotective effects through various mechanisms. Adenosine inhibits neutrophil aggregation and adherence, injury to endothelial cells, reduces ATP depletion during ischemia and repletion of ATP during reperfusion and stimulates myocardial glycolysis (Buja 2005). Adenosine normalises the oxygen supply/demand ratio through vasodilation, antiadrenergic properties and by

inhibiting platelet aggregation. It acts as an antiarrhythmic mediator by accumulating in ischemic cardiomyocytes, which decreases the occurrence of ventricular fibrillation (Donato *et al.*, 2003). Adenosine attenuates myocardial ischemic damage as through systemic as well as indirect effects encompassing heart rate and blood pressure reduction, and increased blood flow due to vasodilation, which could be a possible explanation for its effect on suppression of tachyarrhytmias (Boachie-Ansah *et al.*, 1993; Schreieck *et al.*, 1999).

Adenosine has many direct effects on the cardiovascular system. It can induce vasodilation to restore blood flow and balance the insufficient oxygen supply by activation of adenosine A_2 receptors. Activation of A_{2A} and A_{2B} adenosine receptors causes vasodilation through activation of adenylate cyclase either from endothelial cells or vascular smooth muscle cells via NO release and potassium ATP (K_{ATP}) channels (Donato *et al.,* 2007a). Reports have shown coronary vasodilator effects of adenosine A_{2A} and A_{2B} receptors in various animal models. Adenosine A_{2A} mediated coronary vasodilation occurs in isolated guinea pig heart (Belardinelli *et al.*, 1998), pig heart (Hein *et al.*, 1999) and mouse heart (Morrison *et al.*, 2002). However, some reports have suggested that activation of the adenosine A_{2B} receptor provides vasodilation in rat heart (Hinschen *et al.*, 2003; Rose'Meyer *et al.*, 2003). Another report suggested that coronary vasodilation in mice is caused by adenosine A_{2A} activation whereas in rats, it is caused by adenosine A_{2B} receptor activation (Flood *et al.*, 2001). Adenosine A_1 receptors are found in the heart, and activation of these receptors has indirect anti-adrenergic effects such as a decreases in heart rate, force of contraction and impulse conduction of the heart (Shryock *et al.,* 1997; Ralevic *et al.,* 1998). The effects of

adenosine on SA and AV nodes and atrial myocardium *via* the A_1 receptor include negative chronotropic, dromotropic and inotropic responses. Adenosine acts on the A_1 receptor to depress automaticity (Shryock *et al.,* 1997; Ralevic *et al.,* 1998).

Adenosine inhibits platelet aggregation and has anti-inflammatory effects through inhibition of lymphocyte adhesion to vascular endothelial cells (Hasko *et al.*, 2008). Activation of the A_3 adenosine receptor is involved in preconditioning-mediated cardioprotection (Gessi *et al.*, 2008). Adenosine A_3 receptor activation decreases the work of the cardiac muscle to reduce the oxygen demand of the tissue in times of oxygen deficit and enhances cardioprotection by ATP preservation during ischemia (Cross *et al.*, 2002), also reducing the infarct size (Auchampach *et al.*, 2003).

Table 1.5. Adenosine receptor-mediated physiological effects on various organ systems.

Organ system	Receptor subtypes	Physiological effects References: (Shryock *et al.*, 1997; Jacobson 1998; Ralevic *et al.*, 1998; Sommerschild *et al.*, 2000; Fredholm 2007)
Cardiovascular system (heart and blood vessels)	**A_1AR**	Negative chronotropic, inotropic and dromotropic effects, anti-adrenergic effect, preconditioning-mediated cardioprotection, electrophysiological and metabolic effect, temperature regulation
	A_{2A}AR	Vasodilation, anti-platelet aggregation, anti-inflammatory (inhibition of neutrophil and leukocyte adhesion to the endothelial cells), nitric oxide release (endothelial and VSMC), endothelial cell proliferation and angiogenesis
	A_{2B}AR	Vasodilation, nitric oxide release (endothelial and VSMC)
	A_3AR	Cardioprotection , ischemia preconditioning
Brain	**A_1AR**	Decrease electrical excitability, inhibition of excitatory amino acid (EAA) release
	A_{2A}AR	Anti-inflammatory effect by increase cerebral blood flow, increase excitatory amino acid (EAA) release
Immune system	**A_1AR**	Decrease platelet aggregation
	A_{2B}AR	Allergic response and anti-inflammatory effect
	A_{2A}AR	Mast cell degranulation, wound healing by increase/decrease neutrophil activity
	A_3AR	Allergic responses
Kidney	**A_1AR**	Vasoconstriction, decrease rennin release
	A_{2A}AR	Vasodilation, increase rennin release
GIT	**A_{2B}AR**	Relaxation, increase gastric secretion
Respiratory	**A_1AR**	Bronchoconstriction
	A_{2A}AR	Bronchoconstriction, decrease mucous secretion, anti-inflammatory
	A_{2B}AR	Anti-inflammatory, mast cell- histamine release degranulation,
	A_3AR	Bronchoconstriction

1.6. Adenosine receptor interaction and cross-talk in signalling

Adenosine receptor-mediated cardioprotection involves activation of various subtypes of adenosine receptors. When the activation of one receptor in some way depends on the activation of another receptor, it is known as receptor interaction or cross-talk between receptors. The exact mechanism for adenosine receptor interaction or cross-talk is not fully understood.

1.6.1. Adenosine A_1 and A_2 receptor signalling interaction

Interaction or receptor cross-talk between adenosine A_1 and A_2 receptor signalling has been reported in different pharmacological models. One study demonstrated that the inhibitory effect of adenosine A_1 receptor agonist, N^6-cyclopentyladenosine (CPA), on spike amplitude potential was blocked by the adenosine A_{2A} receptor agonist, 2-*p*-(2-carboxyethy)phenethy-lamino-5'-*N*-ethylcarboxamidoadenosine (CGS21680), in rat hippocampus *in vitro*, and this apparent antagonism was reversed by the adenosine A_{2A} receptor antagonist, ZM241385 (O'Kane *et al.*, 1998). Similarly, the binding affinity of CPA in the rat hippocampal and cortical nerve terminals was reduced in the presence of CGS21680, an effect reversed by ZM241385 (Lopes *et al.*, 1999). The CCPA-mediated antiadrenergic effect has been shown to be inhibited by ZM241385 (Tikh *et al.*, 2006) and adenosine A_{2A} and A_{2B} receptor-mediated vasodilation is negatively regulated by adenosine A_1 receptor activation in isolated mouse hearts (Tawfik *et al.*, 2006). Norton and colleagues have shown that adenosine A_{2A} receptor antagonism enhances adenosine A_1 receptor-mediated antiadrenergic responses in the isolated perfused rat heart (Norton *et al.*,

1999). A point to be noted is that the adenosine receptor cross-talk shown by the above mentioned studies involved antagonistic interactions.

1.6.2. Adenosine A_3 and A_2 / A_1 signalling interaction

Signalling cross-talk between the adenosine A_3 and other adenosine receptors has been reported previously. Stambaugh *et al.* (1997) reported that adenosine A_1 and A_3 receptor activation reduced ischemic damage in chick cardiac ventricular myocytes after prolonged hypoxia, and MRS1191 did not reduce the protective effect of CCPA, suggest that A_1- and A_3-mediated cardioprotective effects appearing to occur independently. There is some evidence that A_{2A} receptor activation plays a role in the effects produced by A_3 adenosine receptor agonists. Maddock and colleagues reported that the A_{2A} receptor antagonist reversed the protective effects of the A_3 agonist Cl-IB-MECA during reperfusion in isolated rat heart, although these authors suggested that this may be related to complex pro-apoptotic and anti-apoptotic effects of A_3 receptor activation (Maddock *et al.*, 2002).

1.6.3. Interaction of adenosine receptors with other receptor systems

Interaction of adenosine receptors with other receptor systems, including opioid (White *et al.,* 1995a; White *et al.,* 1995b; Peart *et al.,* 2003; Peart *et al.,* 2005), dopamine D_1, NMDA (Fredholm *et al.*, 2001), α- and β-adrenoceptors (Tikh *et al.*, 2006) and bradykinin B2 (Xi *et al.*, 2008) receptors have also been reported. However, further details of interaction between adenosine receptors and other receptor systems are beyond the scope of this thesis.

1.7. Ischemic pre-conditioning (PreC) and post-conditioning (PostC)

1.7.1. General definitions

Murray and colleagues first demonstrated the term ischemia PreC in a mammalian model of ischemia-reperfusion damage (Murry *et al.*, 1986). Small episodes of discontinuous sublethal ischemia and reperfusion, which are protective against subsequent lethal episodes of ischemia are called "ischemic pre-conditioning" (Hausenloy *et al.*, 2006). Various survival kinase proteins are involved in the PreC-mediated cardioprotection mechanism. The clinical and practical implications of ischemia PreC are limited, because the timing of myocardial infarction is very difficult to predict. However, this phenomenon may be useful in the case of cardiac bypass surgery, where ischemic events can be predicted (Yellon *et al.*, 2005).

Zhao and colleagues described the term ischemia PostC for the first time, where cardioprotection was induced by the application of small episodes of myocardial ischemia and reperfusion at the start of the reperfusion period (Zhao *et al.*, 2003). Ischemia PostC has been shown to have cardioprotective effects similar to ischemic PreC (Hausenloy *et al.*, 2006). However, clinical application of PostC seems practically much more relevant as reperfusion after a myocardial ischemic attack can be predicted. Therefore, ischemic PostC intervention could be used adjunct to thrombolytic therapy for improved cardioprotection in a clinical situation (Figure 1.10). Further details of PreC and PostC mechanisms are given in subsequent sections.

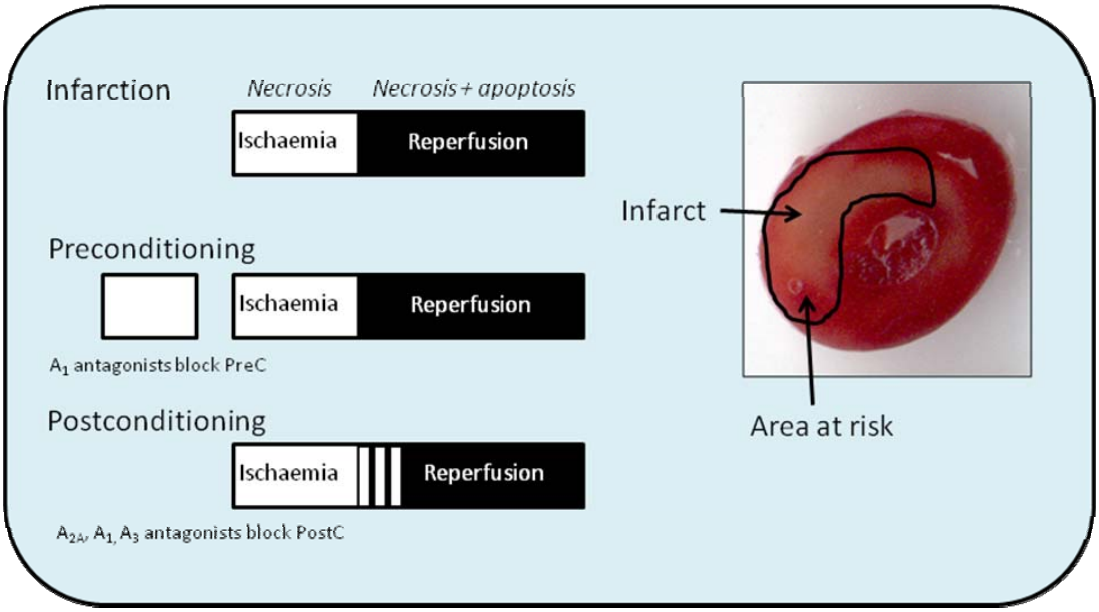


Figure 1.10. Ischemia pre-conditioning and post-conditioning based on the literature presently available.

1.7.2. Ischemia PreC mechanism

Adenosine plays an important role in ischemia PreC. During myocardial ischemia, PreC is mediated in large part by the release of adenosine, as PreC can be abolished using adenosine receptor antagonists and in receptor knockout mice (Morrison *et al.,* 2002; Lankford *et al.,* 2006; Solenkova *et al.,* 2006). Adenosine binds to adenosine receptors and activates various downstream signalling pathways such as PI3K-Akt and ERK1/2. Activation of these signalling pathways leads to the opening of ATP dependent mitochondrial potassium (mitoK_{ATP}) channels (Yellon *et al.*, 2005). Activation of adenosine A_1 and A_2 receptors also leads to antioxidant and anti-inflammatory pathway activation as shown in some studies (Yellon *et al.*, 2003; Yellon *et al.*, 2005). These signal transduction pathways are thought to reduce apoptotic signalling by reducing calcium accumulation,

preserving ATP production inside the mitochondria, diminishing free radical generation, and preventing the opening of the mPTP, which is perhaps responsible for cardioprotection (Yellon *et al.*, 2003; Yellon *et al.*, 2005). When pharmacological agents are used during ischemic conditions, this is known as "pharmacological pre-conditioning", which can be used to target the different elements of signalling pathways (Yellon *et al.*, 2005).

The role of CD73 (ecto-5'-nucleotidase) has been recently identified as an important cardioprotective enzyme (Linden 2001). CD73 plays an important role in production of adenosine from 5'-AMP and increases extracellular adenosine levels (Linden 2001; Jackson *et al.,* 2007), which provides cardioprotection by ischemic pre-conditioning (Linden 2001; Baxter 2002; Burnstock 2002). CD73 regulates vascular barrier function during hypoxic conditions by activating adenosine A_{2A} and A_{2B} receptors (Thompson *et al.*, 2004).

1.7.3. The role of individual adenosine receptors in PreC-mediated cardioprotection

Adenosine receptors play a major role in ischemic pre-conditioning (Ganote *et al.*, 2000; Mubagwa *et al.*, 2001; Nakata *et al.*, 2003). Adenosine A_1 and A_3 receptor activation by endogenous adenosine provides pre-conditioning (Mubagwa *et al.*, 2001). Activation of the adenosine A_1 receptor plays a major role in the phenomenon of pre-conditioning (Headrick 1996). The adenosine A_1 receptor occupancy during the myocardial ischemic period improves post-ischemic functional recovery and protects against vascular dysfunction in the isolated rat (Lasley *et al.*, 1992) and mouse heart (Flood *et al.*, 2002). Adenosine A_1 agonist N^6 cyclohexyladenosine (CHA) improved the postischemic myocardial functions during ischemia reperfusion injury, the effect was abolished by A_1 antagonist DPCPX in isolated

rat heart (Lasley *et al.*, 1992). Endogenous adenosine released during ischemia protects against vascular dysfunction by activation of adenosine A_1 receptor and determines coronary reflow by activation of A_{2A} receptor in ischemia-reperfused isolated mouse hearts (Flood *et al.*, 2002).

The ischemic cardioprotective mechanism induced by adenosine A_1 and A_3 receptors involves activation of PKC, and further activation of several downstream pathways (Yellon *et al.,* 2003; Hausenloy *et al.,* 2006). Pre-ischemic activation of adenosine A_1 and A_3 receptors by selective agonists CPA and IB-MECA respectively gives cardioprotection during ischemia reperfusion injury, the effect is abolished by the MEK/ERK1/2 inhibitor PD98059 and PI3K inhibitor wartmannin in isolated neonatal rat cardiomyocytes (Germack *et al.*, 2005). Endogenous adenosine showed cardioprotection by activation of adenosine A_1 and A_{2B} receptor via Akt-PI3K, ERK1/2 pathways, since the cardioprotective effect is blocked by selective A_1 antagonist DPCPX, A_{2B} antagonist MRS1754, PI3K inhibitor wortmannin and MEK/ERK1/2 inhibitor U0126 in ischemia-reperfused isolated rabbit heart (Solenkova *et al.*, 2006). Adenosine A_{2B} receptor activation by selective agonist BAY606583 shown cardioprotection, which involves activation of PKC and ERK1/2 pathway, the effect was blocked by selective A_{2B} antagonist MRS1754, PKC inhibitor chelerythrine and MEK/ERK1/2 inhibitor U0126 in isolated rabbit heart (Kuno *et al.*, 2007). Adenosine A_1 and A_3 receptor-mediated pre-conditioning balances the oxygen demand and supply ratio during myocardial ischemia. Table 1.6 outlined the various studies used to demonstrate the adenosine receptor involvement in PreC-mediated cardioprotection. Ischemia preconditioning (PreC) have shown a biphasic response of cardioprotection, in

which first phase apparent immediately and last for 1-2 hrs known as classical or early ischemic PreC. After 12-24 hrs the second phase of cardioprotection appears and last for 48-72 hrs is called the "second window of protection or delayed or late ischemic PreC (Kuzuya *et al.,* 1993; Marber *et al.,* 1993; Hausenloy *et al.,* 2010). Adenosine produced during ischemic PreC (Liu *et al.,* 1991) stimulate delayed ischemic PreC by activation of PI3K-Akt, ERK1/2 and JAK, which further activates STAT1/2 and NFkB signaling to provide cardioprotection (Baxter *et al.,* 1994; Hausenloy *et al.,* 2010). Studies show that delayed ischemic PreC was abolished in the presence of the non specific adenosine receptor antagonist 8-SPT, which supports the cardioprotective role of endogenous adenosine in delayed ischemic PreC. Furthermore, the adenosine A_1 receptor agonist CCPA could enhance the cardioprotective effect of delayed ischemic PreC in *in-vivo* rabbit heart ischemia reperfusion injury (Baxter *et al.,* 1994; Dana *et al.,* 1998).

Furthermore, Table 1.6 describes the various adenosine receptors involved in PreC adenosine receptor agonist/antagonist intervention used in the studies, pharmacological models used and the pathways/mechanisms for cardioprotection.

Table 1.6. Cardioprotective signalling during ischemic and pharmacological preconditioning (PreC) through endogenous and exogenous adenosine.

References	Model / species	Preconditioning and/or treatment	Receptor involved and ligands used	Pathway info/ mechanism	Cardioprotection
(Germack *et al.*, 2005)	Neonatal rat cardiomyocyte 4hrs I/18 hrs R	Agonist 15 min pre-ischemia	A_1 (CPA), A_3 (Cl-IB MECA) not A_{2A} (CGS21680)	**MEK/ERK** (PD98059) **PI3K** (Wortmannin) for A_1 and A_3 but not A_{2A} effect	Yes
(Solenkova *et al.*, 2006)	Isolated rabbit heart, CP 30min I/120min R	PreC- one cycle of 5 min I and 10 min R	A_1 (DPCPX) given at 5 or 10 min into reperfusion but NOT 30 min A_{2B} (MRS1754) not A_{2A}	**PI3K** (Wortmannin blocked PI3K at 1st 10 min of R, not after 60 min), **MEK/ERK1/2** (U0126) Protection by A_1 and/or A_{2B}	Yes
(Lankford *et al.*, 2006)	*In vivo* mouse coronary occlusion 45min I/60min R	PreC- 3 cycles of 5 min I and 5min R	A_1KO mice, A_1TG mice (overexpressing A_1 receptor)	Endogenous protection by A_1AR	No PreC in A_1KO mice/Better PreC in A_1TG mice
(Peart *et al.*, 2003)	*In vivo* rat 30min I/90min R	Agonists 10 min pre-ischemia	A_1 (CCPA)	**mitoK$_{ATP}$ channel** (5-hydroxydecanoate) and **ROS** (reactive oxygen species scavenger N-(2-mercaptopropionyl)glycine) dependent mechanism	Yes
(Lasley *et al.*, 1992)	Isolated rat and rabbit heart Constant pressure	Agonist 10 min pre-ischemia	A_1 (CHA), Adenosine	Postischemic functional recovery via **A_1** receptor mechanism	Yes

	(CP), rat 30 min I/ 45 min R; rabbit 60 min I/ 60 min R				
(Lasley *et al.*, 1995)	Isolated rat and rabbit heart 30min I/45min R for rat and 60 min I/60min R for rabbit	Agonist 10 min pre-ischemia	A_1 (PIA), adenosine	Attenuation of myocardial stunning and postischemic functional recovery via **A_1** receptor mechanism	Yes
(Kuno *et al.*, 2007)	Isolated rabbit heart CP, 30 min I/120 min R	PreC- 5 min I/10 min R	A_{2B} (agonist; BAY606583, NECA and antagonist MRS1754) for 20 min starting 5 min before R	**PKC** (inhibitor, Chelerythrine; activator Phorbol12-myristate 13-acetate PMA), **ERK1/2** (inhibitor U0126)	Yes
(Flood *et al.*, 2002)	Isolated mouse heart CP 20-25 min I/ 30min R	Agonist and antagonist prior to and for 15 min following ischemia	A_{2A} (CGS21680, CSC), A_1 (DPCPX)	Endogenous adenosine reduces vascular dysfunction via **A_1** and **A_{2A}** responsible for post-ischemic coronary flow	Yes
(Lasley *et al.*, 2007)	*In vivo* rat heart, 25 min I/120 min R	Agonist 10 min pre-ischemia, antagonist 15 min before agonist	A_1 (CCPA and DPCPX), A_{2A} (CGS21680 and ZM241385), A_1/A_{2A} (NECA), A_3 (MRS1523)	**A_{2A}** and/or **A_{2B}** receptor modulate the **A_1** receptor cardioprotection	Yes
(Regan *et al.*, 2003)	Isolated mouse heart, 30 min I/120 min R, CP	PreC- 20 min pre-ischemia	A_1TG mice (overexpressing A_1 receptor)	Reduced caspase-3 activity and DNA fragmentation, does not involve **PI3K** (LY-294002; PI3K inhibitor)	Yes
(Liu *et al.*, 1991)	In situ and isolated Rabbit heart;	PreC- 5 min I and 10min R Agonist 15 min	A_1 (R-PIA, 8-SPT), A_2 (PD115,199)	Preconditioning induce adenosine level, which activate A_1 receptor	Yes

	30 min I/3 hrs R	pre-ischemia			
(Thornton *et al.*, 1992)	In vivo rabbit hearts; 30 min I/3 hrs R	PreC- 5 min I and 10min R Agonist 15 min pre-ischemia or at Reperfusion	A_1 (R-PIA, CCPA), A_2 (CGS21680)	Preconditioning cardioprotection by A_1 receptor activation	Yes
(Auchampach *et al.*, 1993)	In vivo dog hearts; 60 min I/ 5 hrs R	PreC- 5 min I	A_1 (DPCPX), K_{ATP} antagonist (Glibenclamide)	Preconditioning cardioprotection by A_1 receptor activation involves opening of K_{ATP} channels	Yes

1.7.4. Ischemic PostC definition

Intermittent episodes of ischemia at the start of reperfusion that provide cardioprotection, is known as ischemia post-conditioning. Pharmacological intervention used for PostC-mediated protection is called “pharmacological post-conditioning”.

1.7.5. Role of adenosine receptors in PostC-mediated cardioprotection

The role of adenosine in PostC-mediated cardioprotection has been reported (Vinten-Johansen *et al.*, 2005). PostC cardioprotection involves various mechanisms including activation of reperfusion injury salvage kinases (RISK; PI3K-Akt, ERK1/2, PKC, PGG), adenosine receptors, inhibition of mPTP opening, decreased mitochondrial calcium deposition, antioxidant mechanisms, anti-apoptotic mechanisms, activation of mitoK_{ATP} channels and attenuated inflammatory response (Hausenloy *et al.,* 2004; Vinten-Johansen *et al.,* 2005; Yellon *et al.,* 2005; Hausenloy *et al.,* 2006). PostC-mediated cardioprotection shown by improved post-ischemic cardiac function, Akt and ERK1/2 phosphorylation during ischemia reperfusion injury was abolished by selective A_{2A} antagonist ZM241385 in isolated mouse hearts (Morrison *et al.*, 2007). A study shows that PKG activator 8-(4-chlorophenylthio)-guanosine 3',5'-cyclic monophosphate (CPT) at reperfusion protects ischemic hearts by activation adenosine A_{2B} receptor via PKC, Akt-PI3K, MEK/ERK1/2 and $_{mito}K_{ATP}$ dependent signalling pathways, the cardioprotective effect was abolished by selective antagonist chelerythrine, wortmannin, PD98059 and 5-hydroxydecanoate respectively in isolated rabbit hearts (Kuno *et al.*, 2008). These effects are described in detail in the following section.

Various endogenous autocoids are released during PostC, including adenosine, bradykinin, and opioids. In this condition too, increased concentrations of endogenous adenosine are involved in the cardioprotective mechanism during ischemia-reperfusion damage (Vinten-Johansen *et al.*, 2005). Additionally, adenosine A_1, A_2 and A_3 receptor involvement has been reported in PostC-mediated cardioprotection (Zhao *et al.,* 1993; Kin *et al.,* 2005a).

Intervention at reperfusion is highly desirable in the clinical setting. Given the cardioprotective efficacy of A_1 agonism during ischemia, much attention has focused on A_1 agonist intervention initiated at the time of reperfusion. Unfortunately, outcomes have varied greatly from one study to the next, even in the same species and using similar paradigms (Table 1.7 and 1.8). Adenosine administered at the start of reperfusion or both before ischemia and during reperfusion has been shown to improve post-ischemic dysfunction by activation of A_1 receptors (Donato *et al.*, 2003). Despite some evidence to the contrary (Vander Heide *et al.*, 1996), a number of studies have shown that activation of A_1 receptors by exogenous agonists during reperfusion in isolated rat hearts (Finegan *et al.,* 1996; Lozza *et al.,* 1997; Butcher *et al.,* 2007) or transgenic A_1 receptor over expression in isolated mouse hearts (Matherne *et al.,* 1997) minimizes ischemic contracture and cardiac dysfunction. In contrast, exogenous agonist-mediated A_1 activation had no cardioprotective effect in the hands of other investigators when infused at reperfusion in *in-vivo* rabbit hearts (Thornton *et al.*, 1992; Zhao *et al.*, 1994). There is no obvious reason for this conflicting data, other than the range of agonists and antagonists, the concentrations of each, the range of species used, and the time of onset of agonist treatment. Few attempts have been made to utilise adenosine receptor-mediated cardioprotective mechanisms in the clinic, using

adenosine itself (Mahaffey *et al.*, 1999) or an adenosine A_1/A_2 agonist (Kopecky *et al.*, 2003), but these have resulted in modest protective effects, which were deemed insufficient for further clinical development.

Reports suggest that the activation of 'reperfusion injury salvage kinases' (RISK) such as ERK1/2, p38, p54JNK are not involved in the ischemia preconditioning (Heusch 2009) or postconditioning cardioprotection (Skyschally *et al.,* 2009; Musiolik *et al.,* 2010) in the anesthetized pig ischemia reperfusion injury model. Alternative signaling pathway such as JAK-STAT pathway in the ischemia preconditioning cardioprotection has been reported (Lecour *et al.,* 2005a; Lecour *et al.,* 2005b). Ischemia postconditioning is mediated by SAFE pathway (Survivor Activating Factor Enhancement), which involves the activation of cytokine TNF-α and STAT-3 (Signal Transducer and Activator of Transcription-3) have been reported (Lacerda *et al.,* 2009).

Table 1.7 outlines the various studies used to demonstrate adenosine receptor involvement in the PostC-mediated cardioprotection. Furthermore, Table 1.7 describes the various adenosine receptors involved in PostC adenosine receptor agonist/antagonist intervention used in the studies, the pharmacological models used and the pathways/mechanisms for cardioprotection.

Table 1.7. Cardioprotective signalling during ischemic and pharmacological postconditioning (PostC) through endogenous and exogenous adenosine.

References	Model / species	Postconditioning and/or treatment	Receptor involved and ligands used	Pathway info/ mechanism	Cardioprotection
(Morrison *et al.*, 2007)	Isolated A_{2A}KO mouse heart Constant pressure (CP) 20 min I/30 min R	PostC- 6 cycles of 10 sec R followed by 10 sec I	A_{2A} KO reduced PostC cardioprotection and A_{2A} antagonist (ZM241385)	**pAKT and pERK** reduced in A_{2A}KO PostC compared to WT	Yes
(Baxter *et al.*, 2000)	*In-vivo* rabbit heart, 30 min I/120 min R	Agonist 10 min pre-reperfusion	A_1 (GR79236) did work pre-ischemia	None	No
(Kuno *et al.*, 2008)	Isolated rabbit heart, 30 min I/2hrs R, CP	Agonist 5min pre-reperfusion and continued for 20 min	A_{2B} (MRS1754)/A_1 (DPCPX)	**PKG** activation by (8-(4-chlorophenylthio)-guanosine 3',5'-cyclic monophosphate; CPT), **Akt, PKC** (Chelerythrine), **PI3K** (Wortmannin), **MEK/ERK1/2** (PD98059), **mitoK$_{ATP}$** (5-hydroxydecanoate), **ROS** (reactive oxygen species scavenger N-(2-mercaptopropionyl)glycine) dependent mechanism	Yes
(Donato *et al.*, 2007a)	Isolated rabbit heart Constant flow (CF), 30 min I/30 min R	PostC- 2 cycles of 30 sec I/R	A_1 (DPCPX)	**PostC** cardioprotection by A_1 receptor and K_{ATP} channels (glybenclamide)	Yes

(Liu *et al.*, 2010)	Isolated rabbit heart, 30 min I/120 min R, CP	Agonist 5 min before R and continue for 60 min	A_1/A_2 (AMP579), NECA, A_{2B} antagonist (PSB1115)	**PostC** with AMP579 gives cardioprotection by A_{2B} activation	Yes
(Lasley *et al.*, 2001)	*In-vivo* pig heart, 60 min I/180 min R	Agonist for the first 60 min of R	A_{2A} (CGS21680)	**A_{2A}** activation during reperfusion exerts cardioprotective effect	Yes
(Patel *et al.*, 2009)	*In-vivo* dog heart, 90 min I/ 2.5 hrs or 24 hrs R	Agonist 30 min before R and continued for 2.5 hrs or 24 hrs	A_{2A} (ATL-146e)	**A_{2A}** activation reduced neutrophil adhesion (anti-inflammatory effect)	Yes with 2.5 hrs infusion but not with 24 hrs
(Matherne *et al.*, 1997)	Isolated mouse heart, two groups 20 min I only; 20 min I/ 30 min R, CP	A_1 receptor gene overexpression	A_1TG mice (overexpressing A_1 receptor) and agonist (CPA)	**A_1** overexpression gives increase ischemic tolerance and during reperfusion improve contractile recovery	Yes
(Xi *et al.*, 2008)	Isolated mouse heart, 20 min I/30 min R, CP	PostC- 6 cycles of 10 sec R and 10 sec I	A_1KO mice	Loss of **PostC** induced cardioprotection in A_1KO	Yes
(Kin *et al.*, 2005a)	Isolated mouse heart, 20 min I/30 min R, CP	PostC- 3 cycle of 10 sec R and 10 sec I; 6 cycles of 10 sec R/I	6 cycle PostC improves postischemic systolic and diastolic function	**PostC** cardioprotection by activation of **A_{2A}** and **A_3** but not by A_1. Delayed removal of intravascular adenosine during PostC possibly involved.	Yes
	In-vivo rat heart, 30 min I/180 min R, CP	PostC- 3 cycle of 10 sec R and 10 sec I	A_1 (8-SPT; DPCPX), A_{2A} (ZM241385), A_3 (MRS1523)		
(Butcher *et al.*, 2007)	Isolated mouse heart, 30 min	Agonist 5 min after R for 15 min	A_1 (CPA)	None	Yes

	I/60 min R, CP				
(Peart *et al.*, 2002)	Isolated mouse heart, 20 min I/45 min R, CP	Agonist during entire reperfusion time	A_1 (CPA), 8-SPT, A_{2A} (CGS21680 and ALT146e), A_3 (Cl-IB-MECA), A1TG mice (overexpressing A_1 receptor)	Exogenous adenosine agonist cardioprotection involve activation of **A_3** receptor. A_1 and A_{2A} do not give cardioprotection in this model.	Yes
	Isolated mouse heart, 20 min I/45 min R, and 20 min I/30 min R, CF	Agonist for 3 min at reperfusion	A_1 (Adenosine, 8-SPT), A_{2A} (CGS21680)		
(Jordan *et al.*, 1997)	*In-vivo* dog heart, 60 min I/15, 60, 120 and 180 min R	Agonist infusion 5 min before start of R and continued for 55 min during R	A_{2A} (CGS21680)	**A_{2A}** activation during reperfusion cardioprotection by reduced superoxide generation, reduced adherence of neutrophils to endothelial cell and myocytes, reduced myloperoxide (MPO) activity in the area at risk	Yes
(Finegan *et al.*, 1996)	Isolated rat hearts, 30 min I/ 35 min R, CP	Agonist given either prior to I or only at R for 30 min	Adenosine, A_1 (CHA, DPCPX), A2A (CGS21680)	**A_1** receptor activation give cardioprotection during reperfusion but not A_{2A}	Yes
(Lozza *et al.*, 1997)	Isolated rat hearts, 15 min I/ 60 min R, CP	Agonist infusion started 15 min pre-ischemia and continued for 60 min R	A_1/A_2 (2HE-NECA, CGS21680), NECA), A_1 (CCPA)	**A_1** and **A_{2A}** adenosine receptors give cardioprotection during ischemia and reperfusion	Yes

1.8. Clinical relevance of pharmacological interventions at reperfusion injury

In the clinical setting it is complicated to apply pre-conditioning before onset of ischemic injury due to the unpredictable nature of ischemic occurrences. Cardioprotective strategies have therefore more recently focused on intervention at the time of reperfusion. An additional benefit from this approach is that the treatment can be aimed to correct not only the ischemic damage but also the reperfusion injury. Reperfusion injury has long been recognised as a clinical condition (Buckberg 1986; Buckberg 1987; Piper *et al.,* 1998; Piper *et al.,* 2004) which causes additional injury to the myocardial area at risk by various mechanisms, including reactive oxidative stress, apoptosis, necrosis, and inflammation (Vinten-Johansen *et al.*, 2007).

Discrepancies in the pharmacological PreC- and PostC-mediated cardioprotection could be due to the involvement of various factors such as different species, models, pharmacological agents and concentrations used in various studies. Table 1.8 summarises the various animal species model, adenosine receptor agonists used in the studies which describe the different pharmacological interventions used in ischemia-reperfusion models.

Table 1.8. Pharmacological models in various animal species using PreC and PostC interventions by adenosine A_1, A_{2A}, A_{2B} and A_3 receptor agonists and antagonists

Reference	Study design	Adenosine receptor subtype		
		A_1	**A_{2A}/A_{2B}**	**A_3**
(Cargnoni *et al.*, 1999)	Species Concentration: Agonist Antagonist Time of Drug addition I/R model	Rabbit Heart (Langendorff) **Adenosine**- 1 & 100 μM **CCPA**- 10 nM, 1 μM reported (used 10nM-100 μM) Drug added 10-15 min before Ischemia + Whole time during reperfusion OR only during reperfusion (No difference in the data between two timing of administration) 30 min Ischemia/60 min reperfusion	 **NECA**- 100nM, 5 μM **CGS21680**- 10 nM, 1 μM **He-NECA**- 10, 100 nM reported (used 1 nM-1 μM)	
(Zatta *et al.*, 2006)	Species Concentration: Agonist Antagonist Time of Drug addition	Mice (Langendorff) **CHA**- 50 nM **DPCPX**- 200 nM 10 min prior to ischemia and initial 10 min of reperfusion	 **MRS1754**- 100 nM **SCH58261**- 100 nM	**CI IBMECA**- 100 nM **MRS1220**- 100 nM

	I/R model	20 min Ischemia/ 30 min reperfusion (for coronary vascular function) 20 min Ischemia/45 min Reperfusion (for ventricular contractile response)		
(Flood *et al.*, 2002)	Species Concentration: Agonist Antagonist Time of Drug addition I/R model	Mice (Langendorff) **DPCPX**- 100 & 150 nM 15 min prior to ischemia and 15 min following ischemia 20-25 min Isch/ 30 min reperfusion	 **CGS21680**- 5 nM **CSC**- 100 nM	
(Lasley *et al.*, 2007)	Species Concentration: Agonist- Antagonist- Time of Drug addition I/R model	Rat (In-vivo) **CCPA-** 10 µg/kg **DPCPX** 100 µg/kg (all in i.v. dose) Agonist- 10 min prior to ischemia Antagonist- 15 min prior to ischemia 25 min Ischemia/ 2hrs reperfusion	 **NECA**10 µg/kg **CGS21680** 20 µg/kg **ZM241385**- 1.5 mg/kg	 **MRS1523**- 2 mg/kg
(Lozza *et al.*, 1997)	Species Concentration: Agonist Antagonist Time of Drug addition I/R model	Rat (Langendorff) **CCPA**- 3 nM Started 15 min prior to ischemia and continued for 60 min of reperfusion. 15 min Ischemia/ 60 min reperfusion	 **NECA**- 100 nM **CGS21680**- 10 nM **He-NECA**- 100 nM	

(Morrison *et al.*, 2007)	Species Concentration: Agonist Antagonist Time of Drug addition I/R model	Mice (Langendorff) (A_{2A} KO Mice) Drug given during 30 min of reperfusion (whole time) 20 min Ischemia/ 30 min reperfusion	 **ZM241385**-50 nM	
(Maddock *et al.*, 2001)	Species Concentration: Agonist Antagonist Time of Drug addition I/R model	Guinea-pig (Working heart) **CPA**- 300 nM During 10 at the start of low flow ischemia and maintained throughout reperfusion. 30 min low flow ischemia/20 min reperfusion	 **CGS21680**- 300 nM **NECA**- 300 nM **ZM241385**- 100 nM	
(Tawfik *et al.*, 2006)	Species Concentration: Agonist Antagonist Time of Drug addition I/R model	Mice ((Langendorff) **CCPA**- 1-100 nM **DPCPX**- 50 nM Drugs for 10 min Drugs for 10 min + Antagonist for 5 min No ischemia reperfusion	 **NECA**- 50 nM	
(Kis *et al.*, 2003)	Species	In-vivo rabbit heart		

	Concentration: Agonist Antagonist Time of Drug addition I/R model	**CCPA**- 50 µg/kg **AMP579** (A_1/A_{2A}) 30 µg/kg- bolus; 3 µg/kg for 70 min. 10 min prior to ischemia and continued up to 60 min from reperfusion. 30 min Ischemia/Reperfusion 180 min.	**CGS21680**- 1, 2.5 mg/kg **ZM241385**- 30 µg/kg- bolus; 3 µg/kg for 70 min	
(Morrison *et al.*, 2002)	Species Concentration: Agonist Antagonist Time of Drug addition I/R model	Mice (A_{2A} Ko, WT) **Drugs** for 10 min **Antagonist** 10 min + 5 min with drug No ischemia/reperfsuion	 **NECA**- 100 nM **Alloxazine**- 10 µM	
(Butcher *et al.*, 2007)	Species Concentration: Agonist Antagonist Time of Drug addition I/R model	Mice (Langendorff) **CPA**- 10 µM (for concentration response 10^{-9} to 10^{-5}) **DPCPX**- 10 nM **Drug** added for 15 & 30 min during after 5 min from reperfusion. **Antagonist**- at start of reperfusion 5 min Antagonist then 15 min drug + Antagonist 30 min Ischemia/ 60 min reperfusion		**VCP 102**- 10 µM (A_1/A_3) **MRS1191**- 200 µM

1.9. PreC and PostC mechanisms and the protective role of adenosine receptors

1.9.1. Adenosine A_1 receptor-mediated cardioprotection

The role of the adenosine A_1 receptor in cardioprotection has been studied extensively (Lasley *et al.,* 1992; Lasley *et al.,* 1995; Flood *et al.,* 2002). Activation of the adenosine A_1 receptor induces cardioprotection by various mechanisms including reduction of ATP depletion, stimulation of glycolysis so as to reduce the workload of the heart during ischemia-reperfusion injury, controlling the oxygen demand and supply ratio, increased phosphorylation of ERK1/2, activation of mitoK_{ATP} channels to prevent opening of mPTP, all of which result in reduced apoptosis and necrosis (Figure 1.11) (Ely *et al.*, 1992a; Peart *et al.*, 2007). Studies have shown that treatment with the exogenous adenosine A_1 receptor agonist, RPIA, and CHA before ischemia induced cardioprotection, further suggesting that adenosine A_1 receptor occupancy during the myocardial ischemic period improves post-ischemic functional recovery and protects against vascular dysfunction in isolated rat heart (Lasley *et al.,* 1992; Lasley *et al.,* 1995). Endogenous adenosine effects provided ischemic protection by improving post-ischemic vascular dysfunction by adenosine A_1 receptor activation, and coronary vasodilation by adenosine A_{2A} receptor activation was found by using adenosine A_1 and A_{2A} selective antagonists, DPCPX and CSC, respectively, in an isolated mouse heart ischemia reperfusion model (Flood *et al.*, 2002). Transgenic mouse studies showed that increased over-expression of the adenosine A_1 receptor increased post-ischemic tolerance through increased post-ischemic contractility, but decreased myocardial infarct size and lactate dehydrogenase release (Morrison *et al.*, 2000).

The adenosine A_1 receptor-mediated cardioprotection involved various signalling pathways including the PKC, ERK1/2, PI-3K and Akt pathways (Mubagwa *et al.*, 2001; Jacobson *et al.*, 2006). Treatment with CPA 4 hrs pre-hypoxia and 18 hrs reoxygenation increased phosphorylation of ERK1/2 in neonatal rat cardiomyocytes (Germack *et al.*, 2005). A previous study showed that the exogenous adenosine agonist, NECA, increased ERK1/2 phosphorylation signalling by activation of the A_1 adenosine receptor in CHO cells expressing the human adenosine A_1 receptor (Schulte *et al.*, 2000).

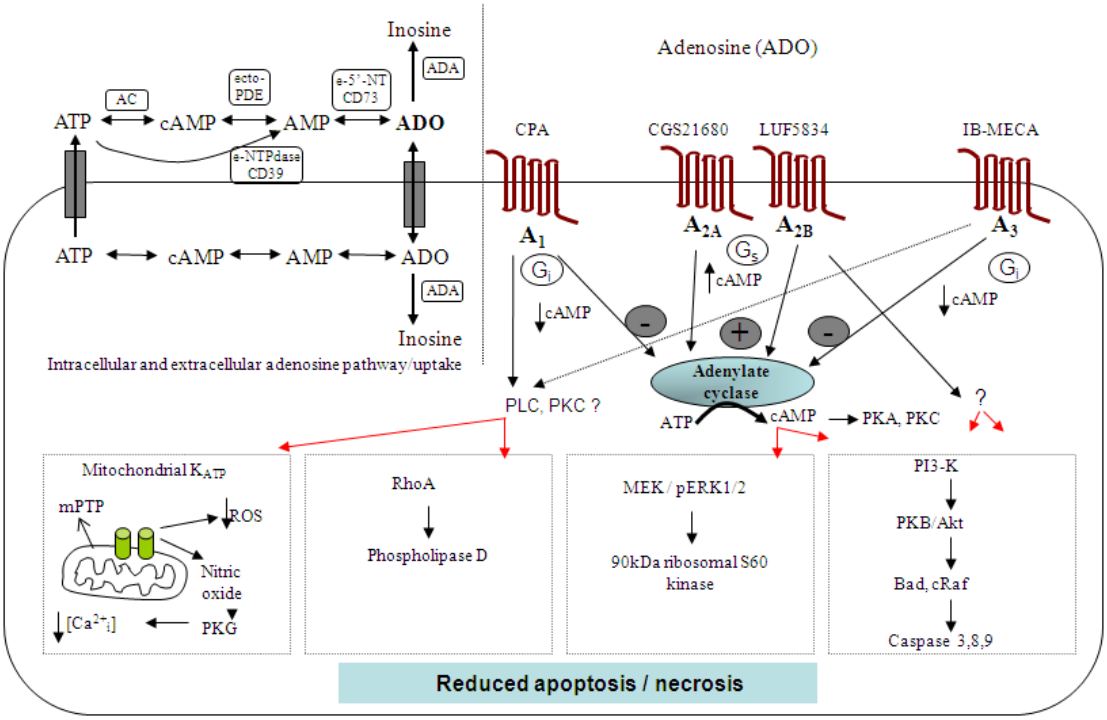


Figure 1.11. Adenosine receptor-mediated signalling and different cardioprotection mechanism based on the literature currently available.

Adenosine A_1 receptor activation increases PKC and PI3K signalling, which contributes to the cardioprotective effect (Peart *et al.*, 2003). Signalling by PI3K activates Akt (PKB), which promotes cell survival by Bad/Bcl protein dissociation and Bad phosphorylation to

provide cardioprotection (Tong *et al.,* 2002; Peart *et al.,* 2003). Furthermore, a review suggested that ERK1/2 promotes cell survival by inhibiting components of the Bad protein (Abe *et al.*, 2000). The activation of PKC during ischemia-reperfusion damage stimulates MAPKinase (ERK1/2, p38MAPK) and K_{ATP} channels (Hausenloy *et al.*, 2004).

PostC cardioprotection involves activation of the reperfusion injury salvage kinase (RISK) pathway, which includes the PI3K-Akt, ERK1/2 and PKC kinase cascade, and is recognised as a pro-survival kinase (Hausenloy *et al.*, 2004). Activation of pro-survival kinase has been shown to induce cardioprotection against reperfusion injury in various animal models (Hausenloy *et al.*, 2004). Various reports have suggested that adenosine A_1-mediated cardioprotection involves MAPKinase (ERK1/2) phosphorylation (Schulte *et al.,* 2000; Germack *et al.,* 2004; Germack *et al.,* 2005). Ischemia PreC mediated cardioprotection involved increased ERK1/2 and Akt phosphorylation, which is blocked by the A_1 and/or A_{2B} selective antagonists DPCPX and MRS1754, respectively, given after ischemia in the isolated rabbit heart ischemia reperfusion model (Solenkova *et al.*, 2006).

1.9.2. Adenosine A_{2A}/A_{2B} receptor-mediated cardioprotection

Adenosine A_{2A} and A_{2B} receptor activation increases cAMP levels by stimulation of adenylate cyclase. Various reports have stated that adenosine A_{2A} activation by CGS21680 provides cardioprotection during reperfusion, in the *in-vivo* open chest pig (Lasley *et al.*, 2001) and *in-vivo* dog models of ischemia-reperfusion damage (Jordan *et al.*, 1997). The adenosine A_{2A} receptor agonist, CGS21680, infusion during reperfusion provided post-ischemic cardioprotection by reducing neutrophil accumulation (shown by reduced

myloperoxide activity) and superoxide production (shown by cytochrome C reduction) in the *in-vivo* dog ischemia reperfusion model (Jordan *et al.*, 1997).

The role of adenosine A_{2B} in cardioprotection has not been extensively investigated. A study has been performed to explore the cardiac effects of adenosine A_{2B} receptor in a adenosine A_{2A} receptor knockout mouse isolated heart (Morrison *et al.*, 2002). This study showed that adenosine A_{2B} receptor activation increases coronary flow and developed pressure in isolated mouse heart. A few studies have suggested a role for the adenosine A_{2B} receptor in ischemic cardioprotection. A study supported the role of the adenosine A_{2B} receptor in ischemic PreC-mediated cardioprotection in the isolated rabbit heart via the Akt and PI3K signalling pathways (Solenkova *et al.*, 2006). Phillip and colleagues have shown that MRS1754 infusion at the start of reperfusion blocked the NECA-mediated infarct size reduction effect (Philipp *et al.*, 2006). Furthermore, the PKC activator phorbol 12-myristate 13-acetate (PMA) increased the PostC cardioprotective effect, which was blocked by the PKC inhibitor, chelerythrin, demonstrated that A_{2B} activation increased the PKC downstream signalling mechanism in rabbit hearts (Philipp *et al.*, 2006).

1.9.3. Adenosine A_3 receptor-mediated cardioprotection

Studies have shown adenosine A_3 mediated cardioprotective effects in isolated rabbit hearts (Tracey *et al.*, 1997) and in the *in-vivo* dog ischemia-reperfusion model (Auchampach *et al.*, 2003). The adenosine A_3-mediated cardioprotective mechanism involves PKC, PI3Kinase, ERK1/2 phosphorylation and K_{ATP} channel activation (Tracey *et al.,* 1997; Tracey *et al.,* 1998; Peart *et al.,* 2007). The protective effect of IB-MECA has been shown

in a number of models including isolated rabbit and mouse hearts (Tracey *et al.,* 1997; Linden 2001; Harrison *et al.,* 2002; Headrick *et al.,* 2003). Ischemic protection has been shown to be reversed by MRS1191 (A_3 selective antagonist; 1300 fold selective for human A_3 vs. A_1 and A_{2A} (Jacobson 1998). Infusion of IB-MECA during ischemia conferred cardioprotection by reducing infarct size in the isolated rabbit heart ischemia reperfusion model (Tracey *et al.*, 1997).

Preclinical research work on adenosine and its receptor subtypes has lead to the clinical trial of various adenosine receptor selective compounds to develop therapeutics for many pathophysiological conditions. In the following section, Table 1.9 describes the different adenosine receptor selective compounds currently used in clinics and undergoing clinical trials for various pathophysiological conditions.

1.10. Adenosine and adenosine agonists in clinical trials

Exogenous adenosine is used in clinical settings to treat supraventricular tachyarrythmias (Sommerschild *et al.*, 2000). Adenosine is also used as a supplement in some cardioplegic solutions. However, use of adenosine in clinics to treat acute coronary ischemia is limited because of significant hemodynamic side effects (hypotension, bradyarrythmia) and short plasma half-life (Sommerschild *et al.*, 2000).

1.10.1. Clinical cardioprotection against ischemic injury

Various studies have demonstrated the cardioprotective effects of adenosine against ischemic injury. The use of adenosine to treat myocardial ischemia in humans has been proposed previously (Kitakaze *et al.*, 1998). There is evidence to suggest that adenosine treatment with low doses of dipyridamole improved tolerance during exercise stress tests by improving chest pain and ST depression in ECG compared to the placebo group (Tommasi *et al.*, 2000). Several studies in humans showed that increased exogenous and endogenous adenosine levels reduced ischemic damage by improved functional recovery and reduced infarct size (Granger 1997; Garratt *et al.,* 1998). Recently, in a large clinical trial "Acute Myocardial Infarction STudy of Adenosine" (AMISTAD; phase-II), intravenous infusion of adenosine (70 μg/mg/min for 3 h) in combination with thrombolytic therapy reduced infarct size in acute myocardial infarction patients (Mahaffey *et al.*, 1999). In another phase-II clinical study (ADMIRE trial), the adenosine A_1/A_2 agonist, AMP579, in conjugation with percutaneous transluminal coronary angioplasty (PTCA) was found to be safe in acute myocardial infarction patients (Kopecky *et al.*, 2003) but not effective. Various adenosine agonists are currently in the clinical development phase and show a promising future for

adenosine and selective adenosine receptor agonist for the treatment of cardiovascular disorders including cardiac ischemia-reperfusion injury (Table 1.9).

In clinical trials involving infusion of adenosine itself (Mahaffey *et al.*, 1999; Ross *et al.*, 2005) or AMP579 (Kopecky *et al.*, 2003) during reperfusion reduced infarct size when administered to treat acute myocardial infarction, although outcomes of the trail were not successes. Another reason for the failure of the ADMIRE trial was that the loading dose of AMP579 did not achieve protective blood levels until 30 min of reperfusion (Liu *et al.*, 2010). However, in another clinical setup adenosine treatment displayed significant reduction in infarct size (Micari *et al.*, 2005) and improved ventricular function in acute myocardial infarction (Marzilli *et al.*, 2000). Again, despite some positive signs, these trials were not successes. It has been recently reported that AMP579 conferred cardioprotection with reduced infarct size by its action on an adenosine A_{2B} receptor in the isolated rabbit heart ischemia reperfusion model (Liu *et al.*, 2010)

Table 1.9. Adenosine receptor agonist in clinical trials (Gao *et al.,* 2007; Elzein *et al.,* 2008).

AR agonist	Affinity of ARs (K_i, nM)				Disease	Clinical phase
	A_1	A_{2A}	A_{2B}	A_3		
Adenosine (Adenocard, adenoscan)	310	700	24,000	290	PVST; cardiac imaging	Marketed
Selodenson (DTI009)	1.1*	306*	ND	ND	AF	II
Tecadenson (CVT-510)	6.5#	2320#	ND	ND	PVST	III
CVT-2759	180*	ND	ND	ND	AF or PVST	ND
Binodenson (MRE0470)	48,000	270	430,000	903	Cardiac imaging	III
Apadenson (ATL-146e) (Adenosine Therapeutics)	77	0.5	ND	45	Cardiac imaging	III
Regadenoson (CVT-3146)	>10,000	290	>10,000	>10,000	Cardiac imaging	III
BAY-60-6583 (Bayer)	>10,000	>10,000	~ 10	ND	Cardiac ischemia	ND
BAY-68-4986 (Bayer)	ND	ND	ND	ND	Angina	III
MRE0094	>10,000	59	>10,000	ND	Diabetic foot ulcers	II
CF101 (IB-MECA)	51	2900	11,000	108	Rheumatoid arthritis	II
CF102 (Cl-IB-MECA)	220	5360	>10,000	104	Colorectal cancer	ND
CF502 (MRS3558)	260	2300	>10,000	0.29	Rheumatoid arthritis	ND

*Rat, # pig

AF: atrial fibrillation; AR: adenosine receptors; ND: not determined or not disclosed; PSVT: paroxysmal supraventricular tachycardia.

1.11. Aims

11.1. Fundamental hypotheses

The fundamental hypotheses underlying the studies reported in this thesis are:

- The presence of endogenous adenosine released during ischemia-reperfusion injury provides a cardioprotective effect through the activation of all four adenosine receptor subtypes.
- Pharmacological intervention *via* adenosine receptor activation during reperfusion provides a clinically relevant model for cardioprotection.
- Adenosine A_1 receptor-mediated cardioprotection during ischemia-reperfusion damage is dependent on cooperative activation of the adenosine A_2 receptor by endogenous adenosine.

11.2. Specific aims

The specific aims of the studies reported in this thesis are:

- Development of a cardiac cell line simulated ischemia model and *ex-vivo* Langendorff perfused isolated mouse/rat heart ischemia-reperfusion injury model to compare and contrast the clinical relevance of adenosine receptor intervention at a genetic, cellular and pharmacological level.
- Determination of the role of endogenous adenosine against ischemic cardioprotection in a cardiac cell line simulated ischemia model.
- Examination of adenosine A_1 and A_3-mediated cardioprotective effects in the presence and absence of endogenous adenosine in a cardiac cell line simulated ischemia model.

- Determination of the cooperative and synergistic cardioprotective effects of the adenosine A_1 receptor requires activation of adenosine A_2 receptors by endogenous adenosine in a cardiac cell line simulated ischemia model.
- Determination of synergistic cardioprotection relating to the additive effect of A_1 and A_2 receptor activation on downstream ERK1/2 phosphorylation signalling using pharmacological and gene knockout intervention in a Langendorff perfused isolated mouse heart ischemia reperfusion model.
- Investigation of the cardioprotective effects of adenosine compounds, including a novel adenosine A_1 agonist, VCP28, and novel adenosine A_3 agonists, VCP103, VCP438, VCP439, VCP485, VCP486 and VCP487; as well as the selective adenosine A_1 and A_3 agonists, CPA and IB-MECA, respectively.

CHAPTER 2

Development of a cardiac cell simulated ischemia model for screening of potential and novel adenosine A_1 and A_3 receptor agonists as a cardioprotective agent

Adenosine A_1 and A_3 receptor agonists-mediated cardioprotection data described in this chapter are published in *Bioorg Med Chem Lett* 2007; 17(19):5437-5441 and *Bioorg Med Chem* 2010; 18:3078-3087 respectively. Sections are renumbered, in order to generate a consistent presentation within thesis.

2.1. Abstract

The aim of the present work was to develop a cardiac cell based ischemia model which could mimic the *in-vivo* ischemic condition, for the preliminary pharmacological screening of a series of novel and selective adenosine A_1 and A_3 agonists made by the Department of Medicinal Chemistry in our faculty. Simulated ischemia was induced by incubating H9c2(2-1) cardiac cells to simulated ischemia (SI) buffer in hypoxic condition (100% N_2 gas) for 4, 6, 8, 10, 12, 16, 20 and 24 hours before assessment of necrosis using propidium iodide (PI; 5 μM). Exposure of cardiac cells to SI buffer for 4, 6 and 10 hrs was unable to increase a significant level of cell death. Incubation of cardiac cells in a SI buffer for 16, 20 and 24 hrs dramatically increased the number of nonviable cells, which was not always reversible in the presence of adenosine A_1 receptor agonist N^6cyclopentyladenosine (CPA). Exposure of cardiac cells to ischemic buffer for 12 hrs significantly increased the number of nonviable cells (40-50% cells were PI positive), the effect reduced in the presence of adenosine A_1 and A_3 receptor agonists CPA and N^6-(3-Iodobenzyl)adenosine-5′-N-methyluronamide (IB-MECA), and novel adenosine A_1 and A_3 agonists VCP28, VCP102 and VCP103, VCP438, VCP439, VCP485, VCP486, VCP487 respectively. A novel, highly selective adenosine A_1 partial agonist VCP28 have shown the cardioprotection at lower nanomolar concentration. In conclusion, we developed a cardiac cell simulated ischemia model to examine the cardioprotective effects of novel adenosine A_1 and A_3 receptor compounds. We evaluated the optimal time of ischemia which would not give complete necrosis to the cardiac cells, and in which ischemic damage can be reversed by the treatment with novel adenosine A_1 and A_3 receptor agonists.

2.2. Introduction

Adenosine is an endogenous ubiquitous autocoid, released due to various physiological and pathophysiological stimuli such as ischemia and reperfusion injury (Ely *et al.,* 1992a; Van Wylen *et al.,* 1992; Headrick 1996). Adenosine exerts its action by activation of adenosine receptor subtypes A_1, A_{2A}, A_{2B} and A_3, which belongs to a G protein coupled receptor (GPCR) family (Fredholm *et al.,* 2001; Linden 2001; Jacobson *et al.,* 2006). Among different adenosine receptor subtypes, adenosine A_1 receptor activation (Lasley *et al.,* 1990; Stambaugh *et al.,* 1997; Peart *et al.,* 2000; Safran *et al.,* 2001) and A_3 receptor activation (Jordan *et al.,* 1999; Maddock *et al.,* 2002; Auchampach *et al.,* 2003; Tracey *et al.,* 2003) have been shown to be cardioprotective in cell and animal model. In recent years, great emphasis has been placed on the development of cardioprotective therapy using adenosine A_1 and A_3 receptor selective agonists as potential therapeutic agents for ischemia and reperfusion injury (see Chapter 1: Introduction, for further discussion).

A series of a novel and selective adenosine A_1 and A_3 receptor agonist has been made by the Department of Medicinal Chemistry in our faculty. For the preliminary pharmacological evaluation of a series of adenosine A_1 and A_3 agonist in our laboratory, a suitable pharmacological model was required, which could mimic the *in-vivo* ischemic condition. The aim of the present work to develop a cardiac cell based model for the preliminary pharmacological screening of the novel adenosine A_1 and A_3 agonists, to select the best candidates for the further detail pharmacological evaluation as a therapeutic target against ischemic injury. To explore the phenotypic change during ischemic condition it is essential

to consider the careful design and development of a cardiac cell simulated ischemia model for the evaluation of novel adenosine compounds for its cardioprotective effect.

Reports suggest that activation of adenosine A_1 and A_3 receptor reduces necrotic cell death in cultured cardiac cell ischemia model (Stambaugh *et al.,* 1997; Liang *et al.,* 1998; Safran *et al.,* 2001). Adenosine A_1 receptor activation reduces myocardial infarct size and improves postischemic functional recovery in isolated rat heart (Lasley *et al.,* 1990; Butcher *et al.,* 2007), mice heart (Matherne *et al.,* 1997; Peart *et al.,* 2000; Flood *et al.,* 2002), *in-vivo* rat heart (Lasley *et al.*, 2007) and *in-vivo* rabbit heart (Thornton *et al.*, 1992). Activation of adenosine A_3 receptor has been shown to protect against ischemia reperfusion injury by reduced infarct size in isolated rabbit heart (Tracey *et al.*, 1997) and *in-vivo* dog heart (Auchampach *et al.*, 2003). In clinical trials, adenosine used as adjunct to the reperfusion thrombolytic therapy in myocardial infarction reduced infarct size (Mahaffey *et al.*, 1999; Ross *et al.*, 2005). Treatment with adenosine A_3 agonist CP-532,903 improved myocardial ischemia and reperfusion injury by activation of sarcolemmal K_{ATP} channels in an *in-vivo* model of infarction and isolated heart ischemia reperfusion model in mouse (Wan *et al.*, 2008). CP-532,903 treatment in mouse thioglycollate-induced inflammation model produced anti-inflammatory effects by inhibition of recruitment of leukocytes into the peritoneum (van der Hoeven *et al.*, 2008).

In the present study, we developed a cardiac cell simulated ischemia model to examine the cardioprotective effects of novel adenosine A_1 and A_3 receptor compounds. We evaluated the optimal time of ischemia which would not give complete necrosis to the cardiac cells,

and in which ischemic damage can be reversed by the treatment with novel adenosine A_1 and A_3 receptor agonists. We compared the cardioprotective effect of novel adenosine compounds with the adenosine A_1 and A_3 receptor selective agonists N^6cyclopentyladenosine (CPA) and N^6-(3-Iodobenzyl)adenosine-5′-N-methyluronamide (IB-MECA), respectively. Adenosine A_1 and A_3 receptor activation involvement was confirmed by using the adenosine A_1 and A_3 receptor selective antagonists 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) and 3-Ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS1191), respectively.

2.3. Materials and methods

2.3.1. Cell culture

The H9c2(2-1) embryonic rat atrial cell line (American Type Culture Collection-ATCC, Manassas, VA, USA) was used for these studies. The cell line was grown in Dulbecco's modified Eagle's medium containing 4 mM L-glutamate, 4.5 g/L glucose, 3.7 g/L sodium bicarbonate, 100 U/ml penicillin and 100 mg /ml streptomycin supplemented with 10% fetal bovine serum (Invitrogen, Mount Waverley, VIC, Australia) in a 5% CO_2 incubator. Cells were used at 60-70 % confluence and plated at the density of $1x10^6$ cells/ml in a 96-well flat-bottomed plate one day prior to assay.

2.3.2. Development and validation of simulated ischemia (SI) model in a cardiac cell line for the preliminary pharmacological screening of the novel adenosine A_1 and A_3 receptor agonists

Pilot studies were performed to develop and establish the optimal conditions to mimic the *in-vivo* ischemic condition in a cardiac cell line. For the development of ischemic condition, cardiac cells were exposed to the SI buffer for different time points in a hypoxic chamber supplied with 100% nitrogen gas. At the start of SI assay normal cell growth media was replaced with the SI buffer (in mM: 137 NaCl, 3.5 KCl, 0.88 $CaCl_2.2H_2O$, 0.51 $MgSO_4.7H_2O$, 5.55 D-glucose, 4 HEPES, 10 2-deoxy-D-glucose and 20 DL-lactic acid (Sigma, Castle Hill, NSW, Australia) plus 2% fetal bovine serum, pH 6.4). We selected eight different time points for the exposure of cardiac cells to the SI buffer in a hypoxic condition (100% N_2 gas atmosphere) at 37^oC. The ischemia model induced both apoptosis and necrosis in cultured H9c2(2-1) cells. Fresh simulated ischemia medium was prepared

for each experiment. At the end of the incubation, cells were stained with propidium iodide (5 μM) to measure the necrotic cell death. Control cardiac cells were exposed to the normal HEPES buffer (pH 7.4). Experimental groups for the development and validation of simulated ischemia model in cardiac cells were as follows: Simulated ischemia (SI) buffer in hypoxic condition for 4, 6, 8, 10, 12, 16, 20 and 24 hours, and non ischemic control group of cells was kept in a normal oxygen incubator (95% O_2 and 5% CO_2 atmosphere) for the same period of time. In the control group HEPES buffer without 2-deoxy-D-glucose and DL-lactic acid was used.

We observed that exposure of cardiac cells for 12 hrs to SI buffer condition causes approximately 40-50% of all cells stained positive to propidium iodide indicating necrosis. We found 12 hrs SI timing was appropriate for the development of best achievable ischemic necrotic cell death, which could not kill all the cardiac cells, but could be reversed pharmacologically. Therefore, we selected the 12 hrs time point as standard for the SI assay for the subsequent preliminary pharmacological screening of the novel adenosine A_1 and A_3 receptor agonists (Figure 2.1 and 2.2, respectively), synthesized in our faculty by the department of Medicinal Chemistry. Experimental groups were as follows: (i) control, (ii) simulated ischemia (SI), (iii) SI + CPA or VCP28 (2e) or VCP102 (5a) or VCP101 (5b) ± DPCPX (10^{-8} M; selective A_1 agonist and a novel A_1 agonist, respectively and 10^{-6} M A_1 antagonist), (iv) SI + IB-MECA or VCP439 (7b) or VCP438 (7c) or VCP486 (9a) or VCP485 (9b) or VCP487 (9c) ± MRS1191 (10^{-8} – 10^{-7} M; selective A_3 agonist and a novel A_3 agonist, respectively and 10^{-6} M A_3 antagonist).

2.3.3. Novel and highly selective adenosine A_1 receptor agonists synthesized in the faculty by the department of Medicinal Chemistry

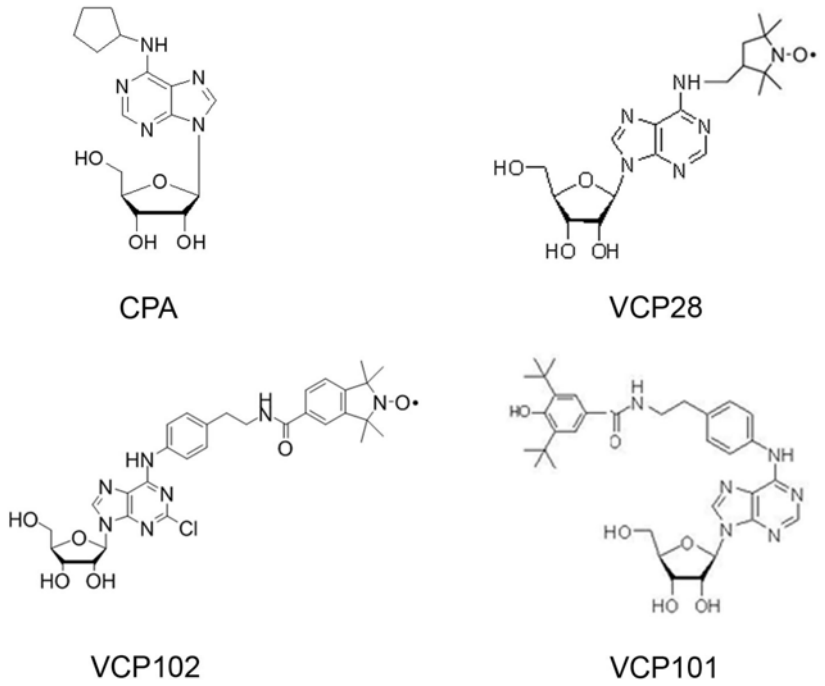


Figure 2.1. Chemical structure of selective and novel adenosine A_1 receptor agonists

Table 2.1. Receptor Affinity of novel adenosine A_1 receptor agonists (*competitive radioligand binding assays using recombinant human adenosine receptors)

Agonist	K_i value for AR (nM)*			
	Hu A_1 AR	Hu A_{2A} AR	Hu A_{2B} AR	Hu A_3 AR
CPA	2.3	794	18,600	72
VCP28 (2e)	50	>10,000	>10,000	21,200
VCP102 (5a)	7	>10,000	1450	23
VCP101 (5b)	32	>10,000	8580	84

2.3.4. Novel and highly selective adenosine A_3 receptor agonists synthesized in the faculty by the department of Medicinal Chemistry

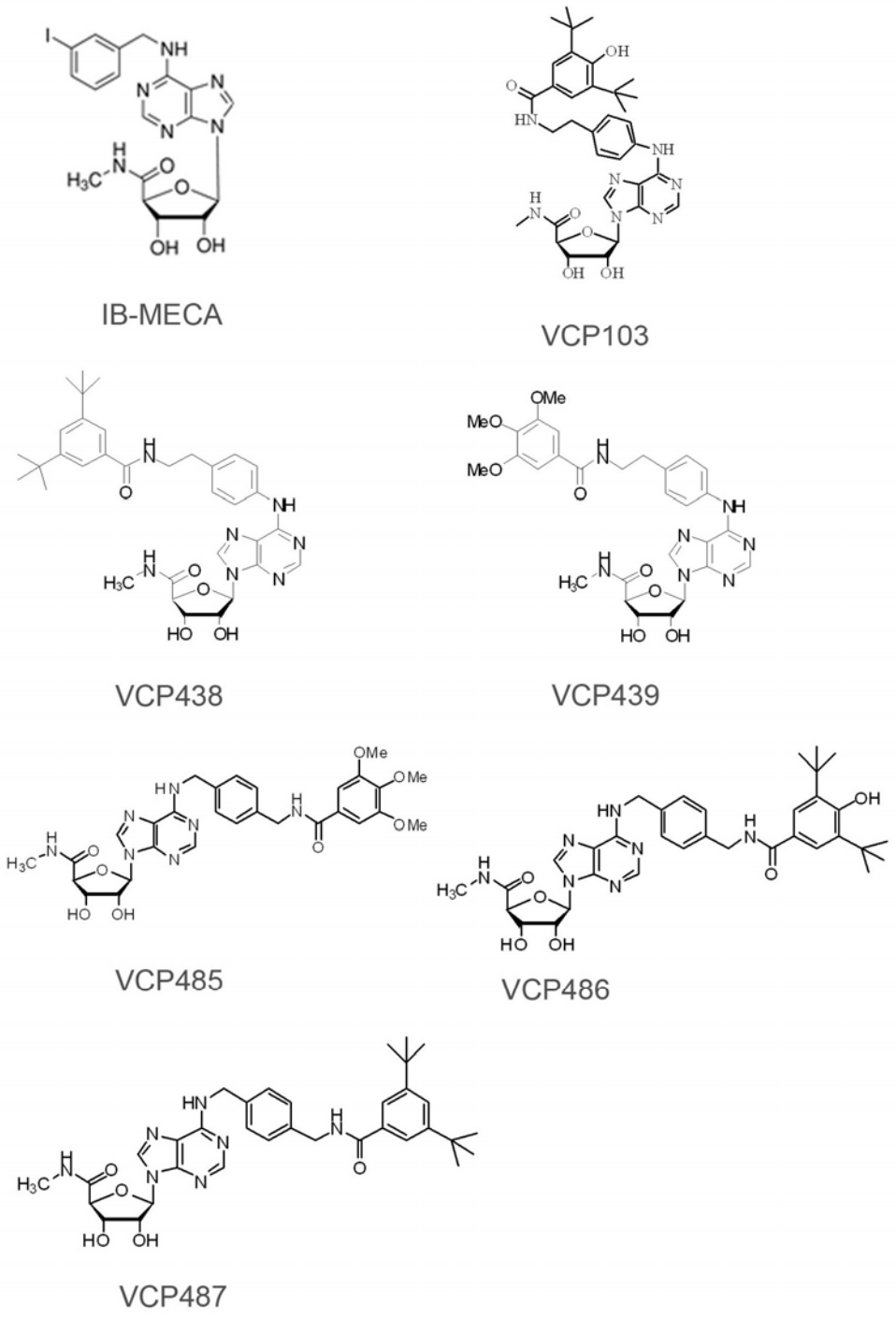


Figure 2.2. Chemical structure of selective and novel adenosine A_3 receptor agonists

Table 2.2. Receptor Affinity of novel adenosine A_3 receptor agonists (*competitive radioligand binding assays using recombinant human adenosine receptors).

Agonist	K_i value for AR (nM)*			
	Hu A_1 AR	Hu A_{2A} AR	Hu A_{2B} AR	Hu A_3 AR
IB-MECA	51	2,900	11,000	1.8
VCP103 (7a)	243	>10,000	6,960	45
VCP439 (7b)	26.2	27% at 10,000 nM	Not disclosed	4.26
VCP438 (7c)	245	>10,000	Not disclosed	2.25
VCP486 (9a)	105	>10,000	Not disclosed	5.63
VCP485 (9b)	76.8	>10,000	Not disclosed	5.04
VCP487 (9c)	128	>10,000	Not disclosed	9.02

2.3.5. General experimental protocol for cell culture

H9c2(2-1) cells were incubated in either normal medium (controls) or hypoxic SI medium. Agonists were diluted freshly with PBS to the appropriate concentrations from stock solutions and added at the start of simulated ischemia. When antagonists were used, they were added 20 min before addition of the agonist. The experimental protocol is summarized in Figure 2.3.

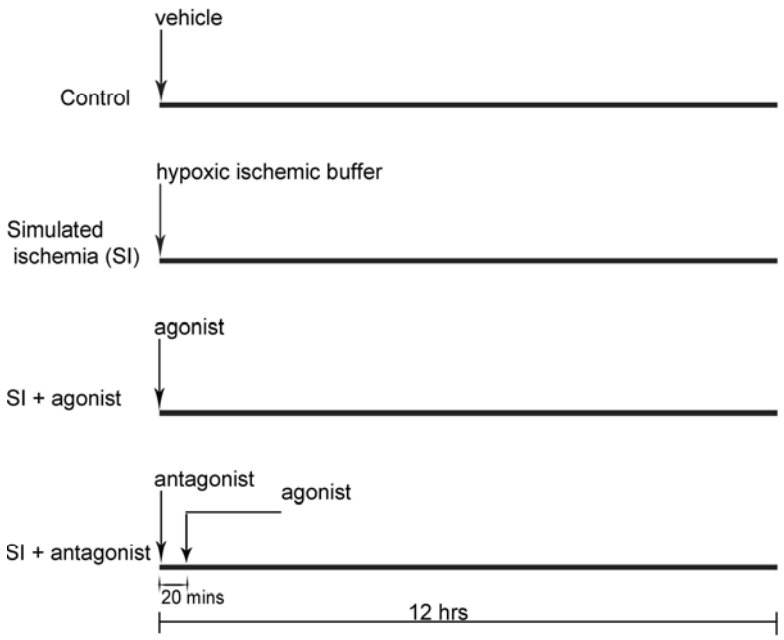


Figure 2.3. The experimental protocol for simulated ischemia assay in a cardiac cell line.

2.3.6. Cell viability (Propidium Iodide) Assay and Imaging of H9c2(2-1) cells

For detection of non-viable cells (necrosis) in all groups**,** a propidium iodide assay was used. At the end of the period of simulated ischemia, 5μM propidium iodide (PI; Sigma, NSW, Australia) was added to each well and incubated for 15 min in the dark. After 15 min images were taken using an inverted fluorescence microscope connected to a SPOT RT camera (Nikon Eclipse TE2000U, Nikon Instruments, Japan) at 535 nm excitation wavelength achieved by DG-4 light box (Shutter Instruments, USA) and 617 nm emission filters. Duplicate wells were used for each experiment, and each experiment was repeated three times (Figure 2.4). From each well 4 images were taken, and propidium iodide positive cells were quantified with using Scion Image Alpha 4.0.3.2 software (NIH Image,

National Institute of Health, USA) then normalized to the SI treatment group (i.e. necrosis was expressed as % cell death with respect to the control SI group using this model).

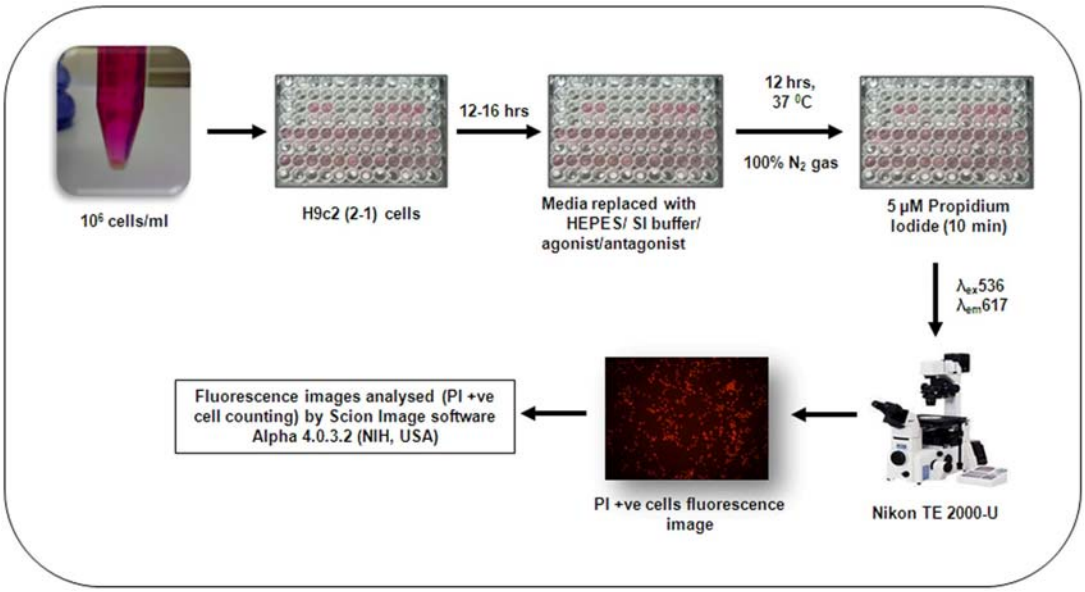


Figure 2.4. Schematic diagram showing the SI assay procedure, imaging and analysis in a cardiac cell line.

2.3.7. Drugs and Preparation of Stock Solutions

N^6cyclopentyladenosine (CPA), N^6-(3-iodobenzyl)-adenosine-5'-N-methylcarboxamide (IB-MECA), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) and 3-Ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS1191) were purchased from Sigma-Aldrich (Castle Hills, NSW, Australia). Novel adenosine A_1 receptor selective agonists VCP28, VCP102, and VCP101 (Figure 2.1) and novel adenosine A_3 receptor selective agonist VCP103, VCP439, VCP438, VCP486, VCP485, and VCP487 (Figure 2.2) were synthesized in the department of Medicinal Chemistry, Monash Institute

of Pharmaceutical Sciences, Monash University, Parkville, Australia. Stock solutions were prepared in dimethyl sylfoxide (DMSO) for all agonists and antagonists containing no more than 0.1% DMSO and stored at -20^{0}C. At the time of experiment stock solutions were freshly diluted in perfusion buffer to make the required concentration.

2.3.8. Statistical analysis

The data were presented as a mean ± SEM. In cell culture simulated ischemia experiments cell viability (PI assay) data were analyzed by one way ANOVA using Bonferroni's post-hoc test for multiple comparisons. For the cell viability assay non-viable cells from SI group were normalized to 100%. $P < 0.05$ was considered statistically significant.

2.4. Results

2.4.1. Development and validation of simulated ischemia (SI) model in a cardiac cell line for the preliminary pharmacological screening of the novel adenosine A_1 and A_3 receptor agonists

Incubation of H9c2(2-1) cells in SI buffer for 12 hrs significantly increased the number of nonviable cells (48.42 ± 8.11% of all cells were stained positive to propidium iodide; PI, Figure 2.7A and 2.9B). The SI group was assigned a value of 100% and other treatment groups were expressed as percentage normalized to the SI group. In the control group, cells incubated in HEPES buffer under oxygenated conditions showed very little cell death (7.36 ± 1.18% with reference to the SI group). Exposure of the cardiac cell to the SI buffer for 4, 6, 8 and 10 hrs did not induce extensive ischemic cell death (4.05 ± 0.75, 9.66 ± 0.83, 9.54 ± 1.19 and 23.62 ± 3.38, respectively, Figure 2.9), the conditions were not sufficient to observe ischemic protection from the adenosine A_1 agonist CPA (Figure 2.5 and 2.6). However, exposure to the SI buffer exposure for a longer duration, i.e.16, 20 and 24 hrs induced greater necrotic cell death (71.09 ± 8.1, 79.83 ± 6.6, 90.28 ± 2.75, respectively; Figure 2.7B, 2.8 and 2.9B), and reversal by CPA. Figures 2.5 to 2.8 show the PI positive cells fluorescence and representative bright field images for the different time point (4 to 24 hrs) exposure of SI buffer to cardiac cells. Based on the pilot experiments, we found 12 hrs SI timing was appropriate for the development of optimal ischemic necrotic cell death, which could not kill all the cardiac cells, but could be reversed. Therefore, we selected 12 hrs time point SI assay for the subsequent preliminary pharmacological screening of the novel adenosine A_1 and A_3 receptor agonists synthesized in our faculty.

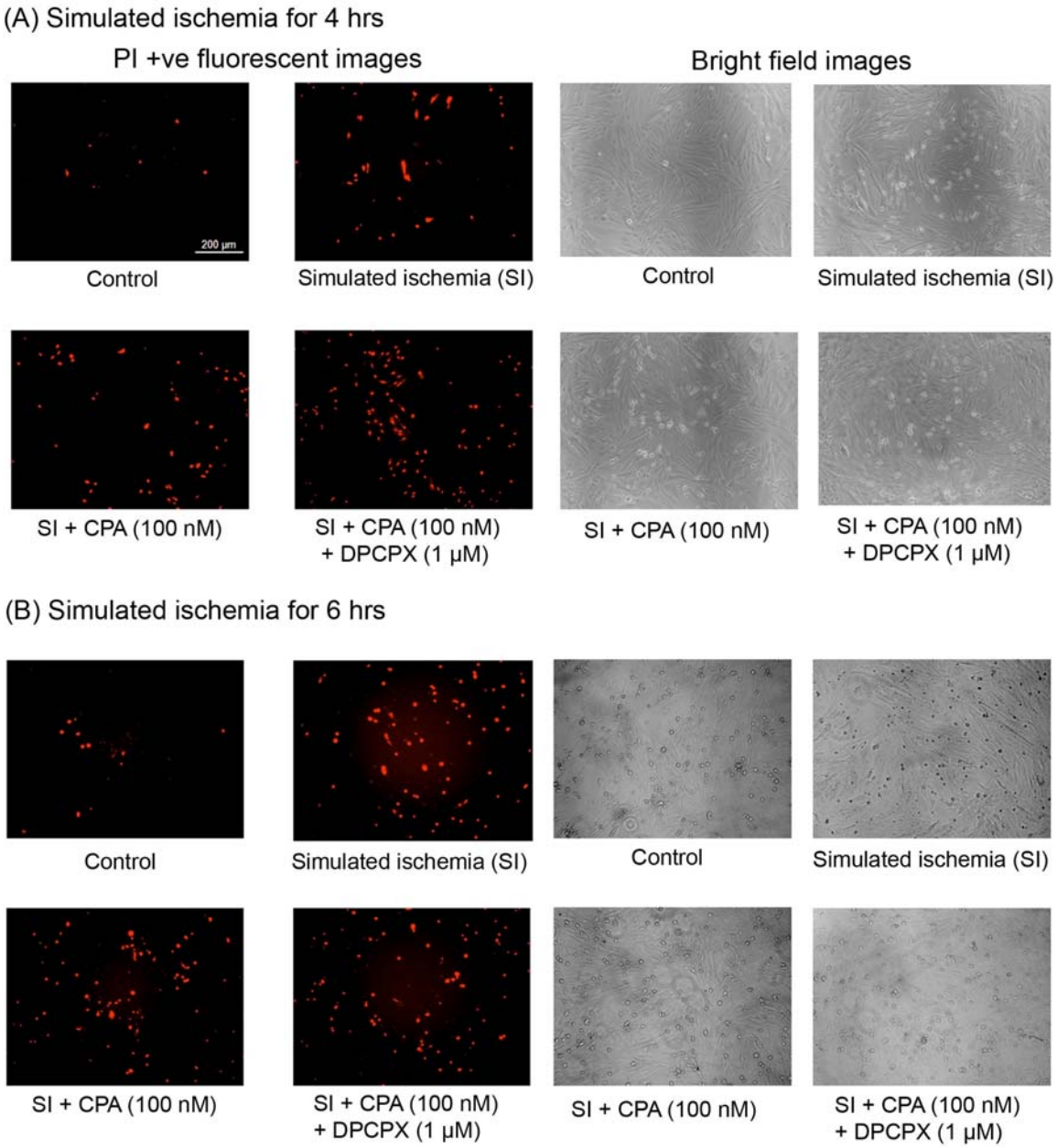


Figure 2.5. Incubation of cardiac cell in SI buffer for 4 (A) and 6 hrs (B). Representative PI positive cells fluorescence images (left side) and respective bright field images (right side) at the end of the SI assay in HEPES buffer (control), SI buffer and in the presence of CPA alone or combination with DPCPX.

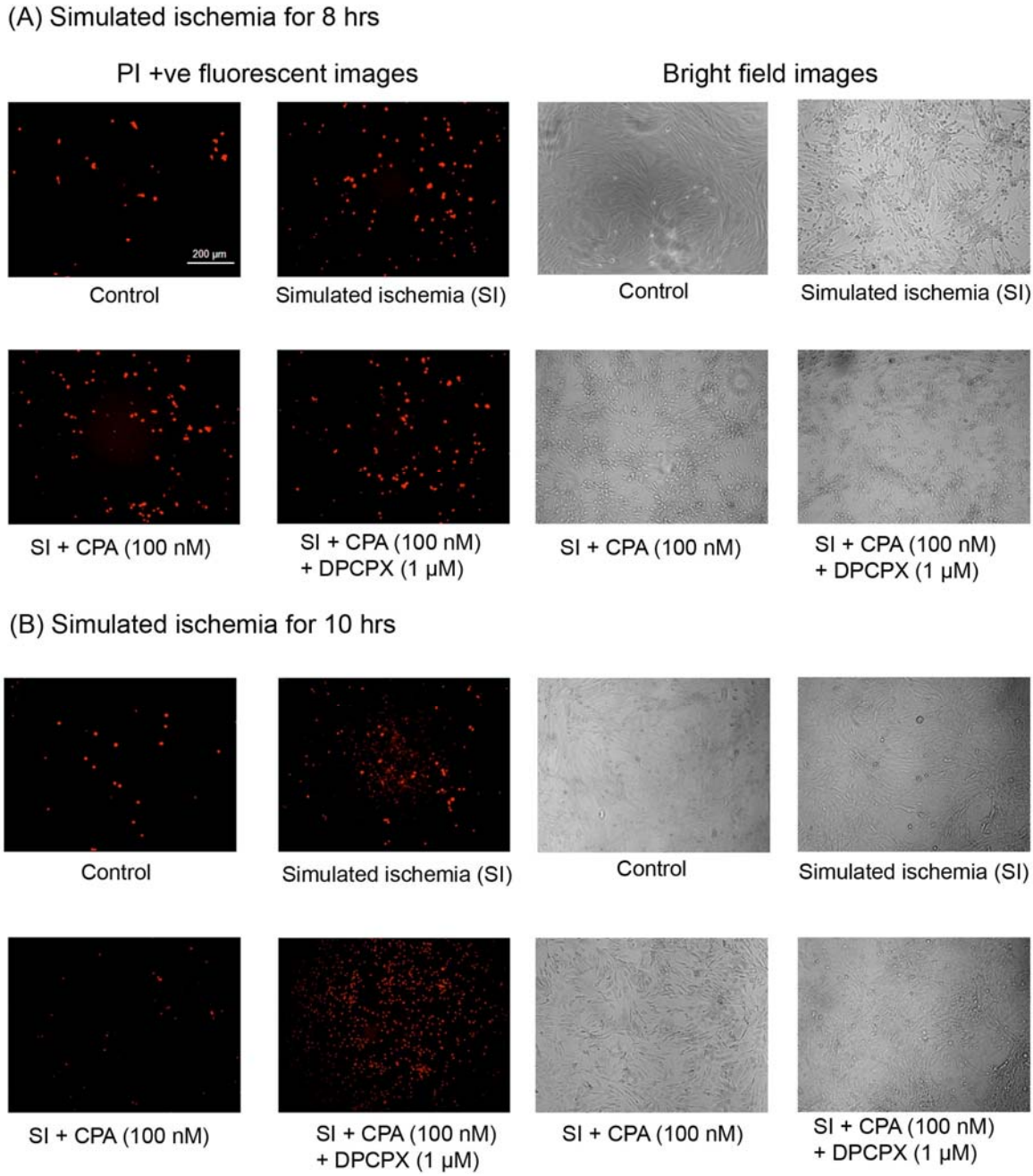


Figure 2.6. Incubation of cardiac cells in SI buffer for 8 (A) and 10 hrs (B). Representative PI positive cells fluorescence images (left side) and respective bright field images (right side) at the end of the SI assay in HEPES buffer (control), SI buffer and in the presence of CPA alone or combination with DPCPX.

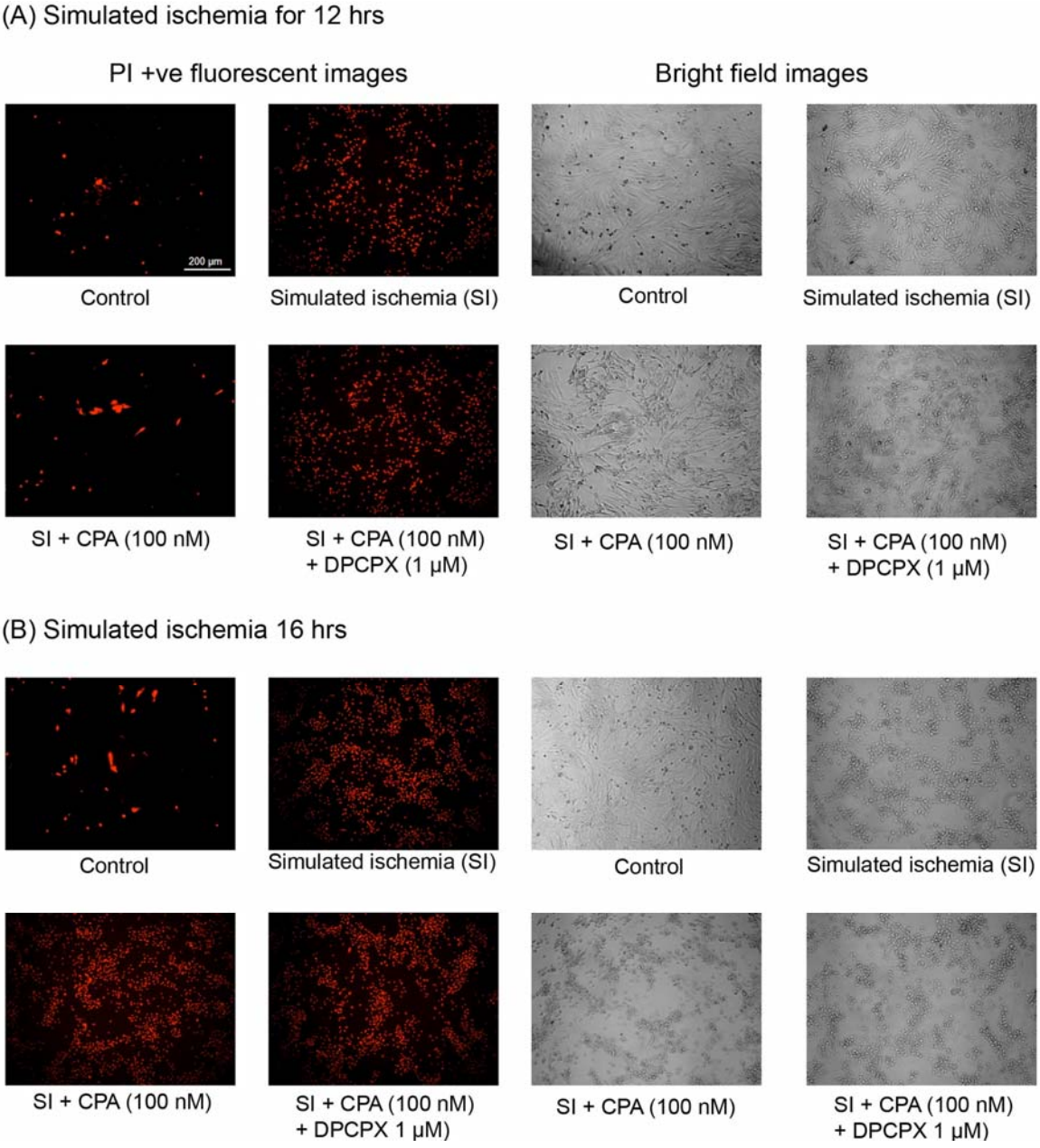


Figure 2.7. Incubation of cardiac cell in SI buffer for 12 (A) and 16 hrs (B). Representative PI positive cells fluorescence images (left side) and respective bright field images (right side) at the end of the SI assay in HEPES buffer (control), SI buffer and in the presence of CPA alone or combination with DPCPX.

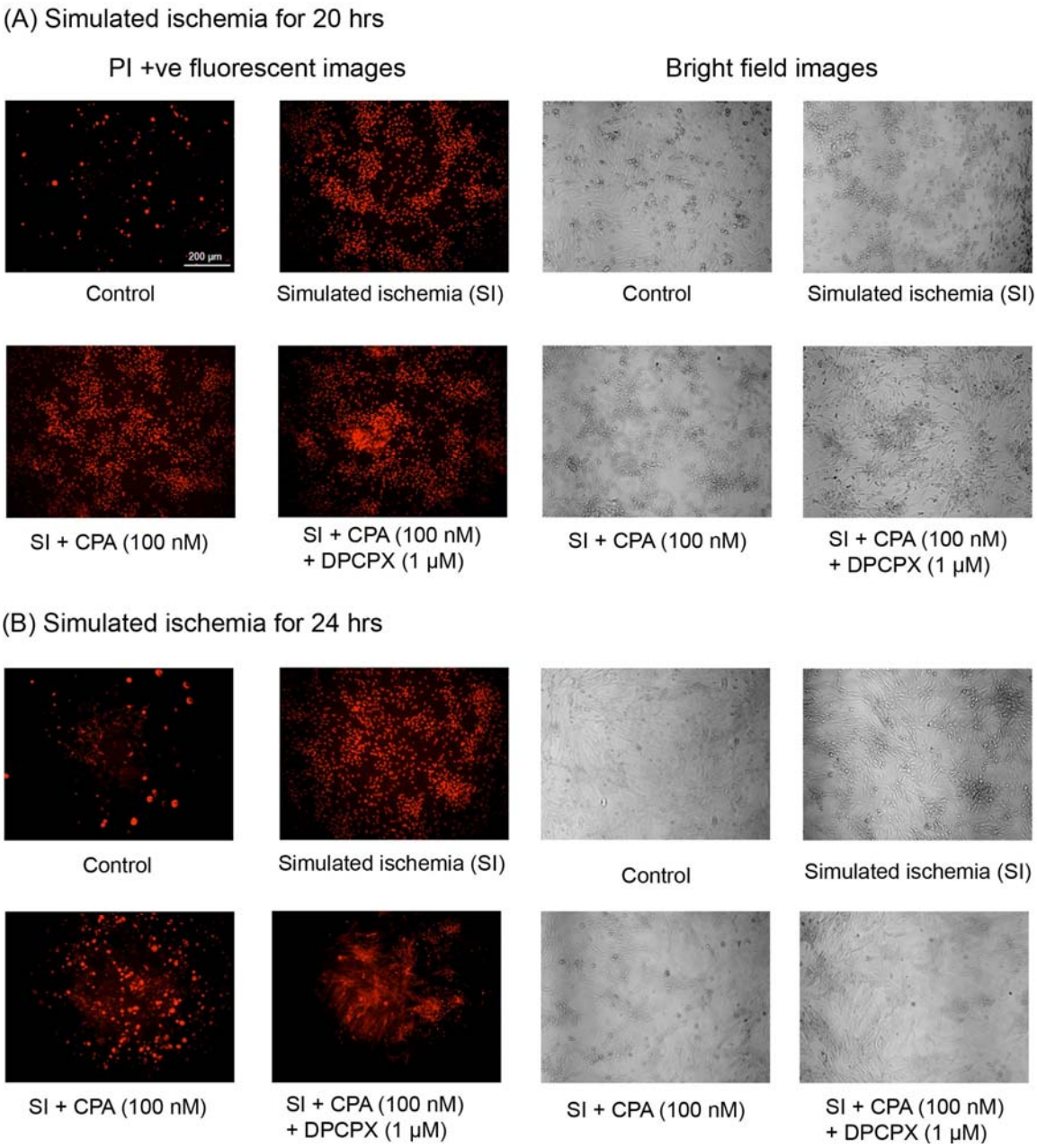


Figure 2.8. Incubation of cardiac cell in SI buffer for 20 (A) and 24 hrs (B). Representative PI positive cells fluorescence images (left side) and respective bright field images (right side) at the end of the SI assay in HEPES buffer (control), SI buffer and in the presence of CPA alone or combination with DPCPX.

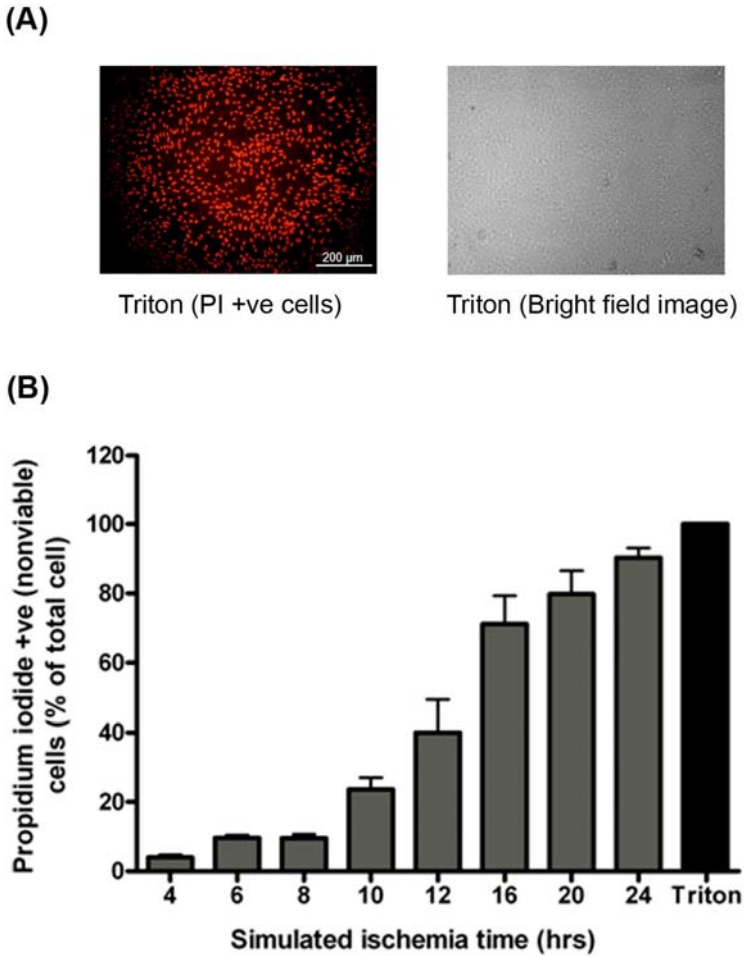


Figure 2.9. Incubation of cardiac cells in SI buffer for increasing time periods (4, 6, 8, 10, 12, 16, 20 and 24 hrs) causes an increase in the proportion of nonviable cells (B). Total cells were determined by treating cells with Triton-X100 (0.8%) to permeabilize all cells, which stained positive for PI (A). The Triton treated group was assigned a value of 100% and different time point exposure of SI buffer were expressed as percentage normalized to the Triton group.

2.4.2. The effect of adenosine A_1 receptor agonists on cell viability during simulated ischemia

Under simulated ischemia conditions, approximately 40-50% of all cells stained positively for propidium iodide (PI), a marker of cell viability (Figure 2.10 – the PI-positive cell number in the simulated ischemia treatment was normalized to 100%). In the control group, cells were incubated in HEPES buffer at oxygenated conditions, and very few nonviable cells were evident. When cells were incubated in the simulated ischemia conditions in the presence of N^6-cyclopentyladenosine (CPA, 10 nM), the number of PI-positive cells was reduced by 86.18 $\pm$ 6.19 %, to a level similar to that seen in cells exposed to normal oxygenated media (control). The series of adenosine receptor agonists tested in this assay all demonstrated cardioprotective properties at the same concentration (10nM). Compound 2e (VCP28; 84.84 ± 3.0 % reduction in dead cell number) demonstrated similar efficacy to CPA at this concentration, whilst compounds 5a (VCP102; 54.74 ± 19.89 % reduction in dead cell number) and 5b (VCP101; 73.24 + 7.8% reduction in dead cell number) were less efficacious. These protective effects of CPA and analogs 2a, 5a and 5b were all significantly reduced in the presence of the adenosine A_1 receptor antagonist 1,3-dipropyl, 8-cyclopentyl xanthine (DPCPX), over the range of agonist concentrations of 10-1000 nM (ANOVA, $P<0.05$).

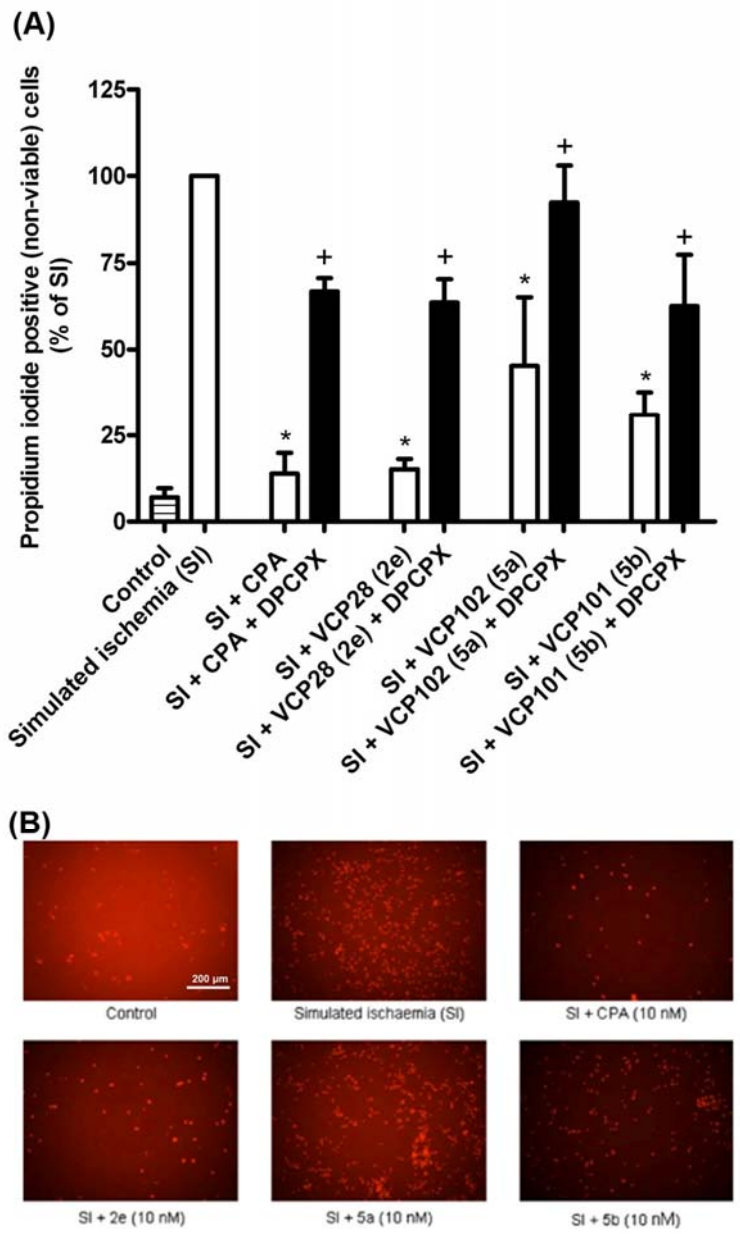


Figure 2.10. (A) The effects of adenosine receptor agonists on a cell model of ischemia. Cells were grown in either normal medium (control) or hypoxic simulated ischemia medium (all other treatments) for 12 hrs. Propidium iodide exclusion was then used to determine the number of viable cells, and cell death calculated for each treatment, with the simulated ischemia treatment normalised to 100% (mean ± SEM, n = 3). (B) Representative images from cells incubated as described in Figure 2.10A.

2.4.3. The effect of adenosine A_3 receptor agonists on cell viability during simulated ischemia

When cells were incubated in the simulated ischemia conditions in the presence of the prototype A_3 agonist IB-MECA, the number of PI-positive cells was reduced by 55.07 $\pm$ 6.7 % (n=4, P<0.05). The series of adenosine receptor agonists tested in this assay all demonstrated cardioprotective properties at the same concentration (10 and 100 nM, Figure 2.11). All five novel A_3 agonists tested showed equal or greater protective activity than IB-MECA, with compound 9c reducing cell death in this model by 68.02 $\pm$ 3.6 % (n=4, P<0.05). There were no significant differences between the level of protection conferred by the different analogues tested (n=4, P<0.05). The protective effects of IB-MECA and analogues 7a (VCP103), 7b (VCP439), 7c (VCP438), 9a (VCP486), 9b (VCP485) and 9c (VCP487) were all significantly reduced in the presence of the A_3 antagonist 3-ethyl 5-benzyl-2-methyl-6-phenyl-4-phenylethynyl-1,4-(+)-dihydropyridine-3,5-dicarboxylate (MRS-1191) at 100 nM agonist concentration (n=4, P < 0.05, Figure 2.11). These data indicate that the adenosine receptor analogues evaluated have significant A_3-mediated cardioprotective effects at nanomolar concentrations.

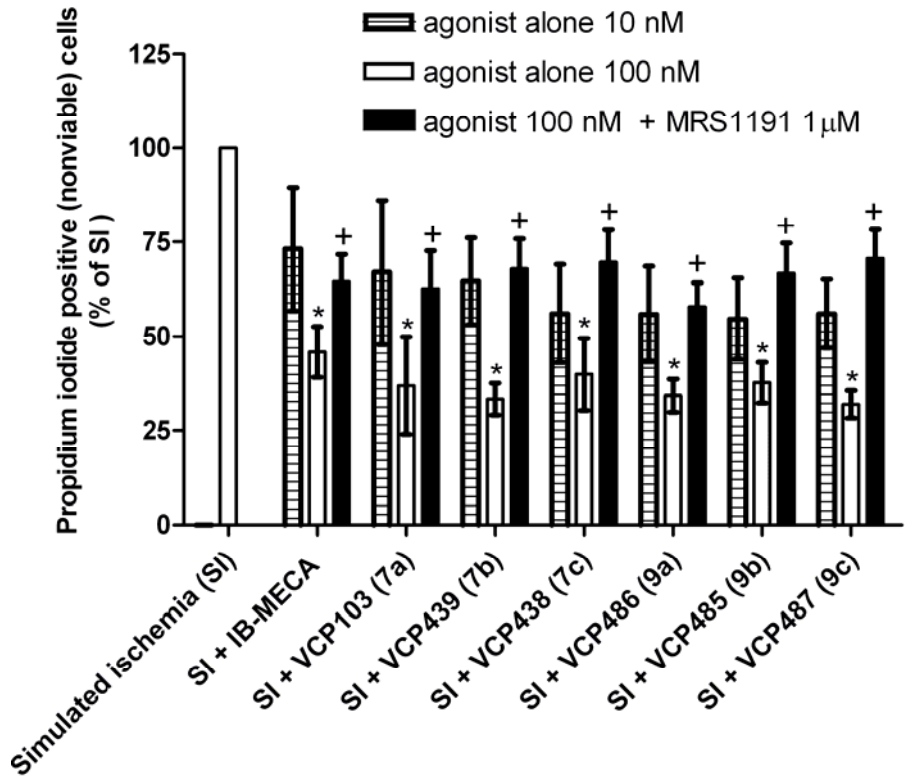


Figure 2.11. Cardioprotection exerted by novel A_3 agonists in a cell culture ischemia model. Cells were grown in either normal medium (control) or hypoxic simulated ischemia medium (all other treatments) for 12 hrs. Propidium iodide exclusion was then used to determine the number of viable cells, and cell death calculated for each treatment, with the simulated ischemia treatment normalised to 100% (mean ± SEM, n= 4). * indicate a significant difference to the SI group, + indicate a significant difference to the relevant agonist treatment without antagonist.

2.5. Discussion

In the present study we evaluated necrotic cell death in a rat cardiac cell line H9c2(2-1) when cells were exposed to SI buffer for 4, 6, 8, 10, 12, 16, 20 and 24 hrs to create a simulated ischemic condition. We determined the effect of exposure of SI buffer for increasing time periods on cardiac cell viability as a measure of necrosis; the optimal time period was used for preliminary pharmacological screening of novel adenosine A_1 and A_3 receptor selective compounds for the evaluation of their cardioprotective effect during ischemic injury. Exposure of cardiac cells to SI buffer for 4, 6 and 10 hrs was unable to increase a significant level of cell death, and no significant difference in the number of nonviable cells were observed between the ischemic cells alone or in presence of adenosine A_1 agonist CPA. Incubation of cardiac cells in a SI buffer for 16, 20 and 24 hrs dramatically increased the number of nonviable cells, which was not always reversible in the presence of CPA. Exposure of cardiac cells to ischemic buffer for12 hrs significantly increased the number of nonviable cells, and this effect was reduced in the presence of CPA.

A number of *in-vitro* simulated ischemia models in cardiac cell culture have been described (Liang 1996; Strickler *et al.,* 1996; Chapman *et al.,* 2002; Bonavita *et al.,* 2003; Tantini *et al.,* 2006), most of these cell models result in irreversible myocyte injury. Various studies used H9c2(2-1) cardiac cell and reported the SI buffer exposure to cell line for 40 min (Vitadello *et al.*, 2003), 3 to 4 hrs (Gordon *et al.*, 2003; Jung *et al.*, 2004), 8 hrs (Bonavita *et al.,* 2003; Moon *et al.,* 2004; Malliopoulou *et al.,* 2006; Tantini *et al.,* 2006), 12 hrs (Zhao *et al.,* 1998; Bonavita *et al.,* 2003; Tantini *et al.,* 2006), 16 hrs (Chapman *et al.*,

2002), and 24 to 48 hrs (Bonavita *et al.*, 2003) to induce simulated ischemia in a cell culture model (see Table 2.3). Due to this large range in the literature, a pilot study in our own laboratory was performed. To inform our pilot studies, we considered different simulated ischemia models used in the H9c2(2-1) cardiac cells, adult rat (Zhao *et al.,* 1998; Gordon *et al.,* 2003), rabbit (Ohata *et al.*, 1994), and human cardiomyocytes (Carroll *et al.*, 2000) for the ischemic effect on the different species. We observed that exposure of cardiac cells to SI buffer for 12 hrs resulted in optimal cellular injury compared with the shorter or longer incubation times. The presence of the adenosine A_1 agonist CPA provided cardioprotection in this model, suggesting that our SI model caused some degree of cell death that could be reversed by the use of an A_1 agonist, which is in agreement with the ischemic studies, using H9c2(2-1) (Malliopoulou *et al.*, 2006) and human atrial cardiomyocyte (Carroll *et al.*, 2000) for the simulated ischemia model.

We induced simulated ischemic condition in a rat cardiac cell line using a HEPES based nonlethal SI buffer containing 2-deoxy-D-glucose and DL-lactic acid to induce metabolic inhibition and increase lactic acid level with pH decreased to 6.5, which mimic the *in-vivo* ischemic condition. Other studies have reported the use of lethal SI buffer containing sodium cyanide (NaCN) and hydrogen peroxide (H_2O_2) as an ingredient to induce ischemic and hypoxic condition in H9c2(2-1) cardiac cells (Jung *et al.*, 2004) and adult rabbit cardiomyocyte (Ohata *et al.*, 1994). The cellular insults by lethal chemicals such as sodium cyanide and hydrogen peroxide are less similar to human ischemia than our model.

The result of the present study should be interpreted with a caveat that the adenosine A_1 and A_{2A} antagonist DPCPX and ZM241385 respectively, have a relatively high binding affinity for the adenosine A_{2B} receptor as well in various rodent species, which has been reported recently (Auchampach *et al.,* 2009). The adenosine A_{2B} antagonist MRS1754 (200 nM) used in the present study has shown some binding affinity for the adenosine A_1 receptor in rat (Table 1.2 B). In hindsight, a lower concentration of DPCPX and MRS1754 would have been appropriate.

Activation of adenosine A_1 and A_3 receptor agonist before simulated ischemia by CCPA and IB-MECA, respectively has been shown to reduce cell death in chick ventricular myocyte model (Strickler *et al.,* 1996; Stambaugh *et al.,* 1997) and rat cardiomyocyte (Safran *et al.*, 2001). In the present study, we observed a reduced number of nonviable cells, when cardiac cells were treated with adenosine A_1 and A_3 agonist CPA and IB-MECA, respectively. The presence of the adenosine A_1 and A_3 selective antagonists DPCPX and MRS1191 respectively abolished the cardioprotection. The data from the present study suggest that adenosine A_1 and A_3 receptor activation before ischemia gives cardioprotection in our cardiac cell line ischemia model.

Table 2.3 Various **s**imulated ischemia (SI) model in cardiac cells

Cardiac cell type	Cell density	Ischemic time period	SI buffer composition	Hypoxic condition	Cell death measurement	References
H9c2(2-1) rat cardiac cells	10, 000 to 12, 000 cells/cm^2	8, 24, 48 and 72 hrs	Serum-deficient DMEM saturated with 5% CO_2/95% N_2	Anaerobic workstation (BugBox, Jouan, France) 5% CO_2/95% N_2 at 37^0C	Trypon blue exclusion	(Bonavita *et al.*, 2003)
Human atrial cardiomyocyte	3 x10^5 cells/cm^2	3 hrs	(mM): NaCl, 135; KCl, 5.4; $MgCl_2$, 0.5; NaH_2PO_4, 0.33; HEPES, 5.0; $CaCl_2$, 1.8; and Na^+-lactate, 20; Deoxydeglucose 20; pH 6.3	Hypoxic chamber which was evacuated and then percolated with an anoxic gas mixture (95% argon, 5 % CO_2)	Propidium iodide (PI) assay, LDH assay	(Carroll *et al.*, 2000)
Rat ventricular cardiomyocyte	Not disclosed	36 min	(mM): NaCl, 135; KCl, 5.4; $MgCl_2$, 0.5; NaH_2PO_4, 0.33; HEPES, 5.0; $CaCl_2$, 1.8; and Na^+-lactate, 20; pH 6.8; bubbled with 100% N_2 for >45 min	100% N_2 at 37^0C	Lactate level	(Lu *et al.*, 2005)
H9c2(2-1) rat cardiac cells	Not disclosed	16 hrs	Serum and glucose-free DMEM, containing 20 mM 2-deoxyglucose	95% N_2/5% CO_2, at 37^0C	Nucleosome ELISA	(Chapman *et al.*, 2002)
Neonatal rat ventricular cardiomyocytes	1–1.5 x 10^6 cells	2, 4, 6, 8, 10, and 12 hrs	(mM): 118 NaCl, 24 $NaHCO_3$, 1 NaH_2PO_4, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 0.5 Sodium EDTA, 20 Sodium lactate, and 16 KCl, pH 6.2; and pre-gassed with 5% CO_2,	Purpose-built ischemia chamber and incubated at 37 °C in 5% CO_2, 95% argon	Trypon blue exclusion and LDH assay	(Zhao *et al.*, 1998)

			95% argon			
H9c2(2-1) rat cardiac cells	1x10^5 cell	10, 30, 60, 120, and 180 min	mM; 106, NaCl; 4.4 KCl; 1 $MgCl_2$.6H2O; 38 $NaHCo_3$; 2.5 $CaCl_2$; 20 2-deoxyglucose; 1 NaCN; pH 6.6	Humidified chamber 95% air, 5% CO_2 at 37^oC	XTT (Sodium 3'-[1-phenylaminocarbonyl)-3-4-trtrazolium]-bis [4-methoxy-6-nitro] benzene sulfonic acid) assay, LDH assay	(Jung *et al.*, 2004)
H9c2(2-1) rat cardiac cells	6000–8000 cells/cm^2	8 hrs	Serum-free, glucose-free DMEM without sodium pyruvate	Anaerobic GasPak pounches (Becton Dickinson) and incubated at 37 ^0C	PI assay	(Malliopoulou *et al.*, 2006)
H9c2(2-1) rat cardiac cells	2x10^5 cells/well	8 hrs	Serum-free, glucose-free DMEM saturated with N_2 gas for 1 h, 37 ^oC	Anaerobic chamber (Forma Scientific, Marietta, OH, USA) maintained at 37 ^0C with humidified atmosphere of 5% CO_2, 10% H_2 and 85% N_2	PI assay , Terminal deoxynucleotidyl transferase UTP nick end labeling (TUNEL) assay	(Moon *et al.*, 2004)
Adult rabbit cardiomyocyte	15,000/cm^2	1, 2, 3 and 4 hrs	(mM): 110 NaCl; 5 KCl; 1.25 $CaCl_2$; 0.5 Na_2HPO_4; 0.5 KH_2PO_4; 1 $MgSO_4$; 20 HEPES; 5 NaCN; 20 2-deoxy-D-gluocose, pH 6.2	Airtight perfusion chamber infused with anoxic suspension of submitochondrial articles (1mg protein/ml), 5 mM succinate, 20 mM 2-deoxy-D-glucose	PI assay	(Ohata *et al.*, 1994)

H9c2(2-1) rat cardiac cells	Not disclosed	2, 3,4, 6, 12, 24, 48 hrs	Serum-deficient medium in an saturated with 5% CO_2–95% N_2	Anaerobic workstation (BugBox, Jouan, France), 5% CO_2–95% N_2 at 37 °C	Trypon blue exclusion	(Tantini *et al.*, 2006)
H9c2(2-1) rat cardiac cells	1×10^6 cells	40 min	(mM): 118 NaCl, 24 NaHCO3, 1 $NaH_2PO_4H_2O$, 1.2 $MgCl_2$, 2.5 $CaCl_2 2H_2O$, 0.5 $NaEDTA 2H_2O$, 20 sodium lactate, and 16 KCl, pH 6.2 pre-gassed with 100% N_2 for 40 min	Ischemia chamber, incubated in 100% N_2 at 37°C	LDH assay	(Vitadello *et al.*, 2003)
Adult rat cardiomyocyte	3-4 x 10^5 cells/35mm well	4 hrs	(mM) 137 NaCl, 3.5 KCl, 0.88 $CaCl_2.2H_2O$, 0.51 $MgS0_4$. $7H_2O$, 5.55 D-glucose, and 4 HEPES and 2% FCS supplemented with 10 mM 2-deoxy-D-glucose and 20 mM DL-lactic acid, pH 6.5	95% O_2/ 5% CO_2 at 37°C	LDH assay	(Gordon *et al.*, 2003)

In the present study we used a series of novel dual acting antioxidant A_1 adenosine receptor agonists, which were both potent and selective via receptor binding assays (Gregg *et al.*, 2007). Among the compounds synthesized were VCP28, which had high affinity (K_i = 50 nM) and good selectivity ($A_3AR/A_1AR \geq 400$), and VCP102 with higher affinity still (K_i = 7 nM), but lower selectivity ($A_3AR/A_1AR = \sim 3$; Table 2.1). These compounds include an antioxidant group without deleterious effects in terms of adenosine receptor selectivity. All of the N^6-nitroxide compounds (VCP28; 2e and VCP102; 5a) had sub-micromolar K_i values at the adenosine A_1 receptor and good selectivity versus adenosine A_{2A} and the A_{2B} receptor. VCP28 and VCP102 have antioxidant moiety (nitroxide radical) attached at the N^6 position of the purine ring without loss of selectivity for the adenosine A_1 receptor and cardioprotective effect. A recent report suggest that VCP102 administration during reperfusion injury reduces infarct size and improves postischemic cardiac functional recovery in isolated mouse heart model (Butcher *et al.*, 2007). VCP28 is further characterised in this thesis in Chapter 5 (Urmaliya *et al.*, 2010a).

The novel adenosine A_3 agonists have a key component of adenosine A_3 receptor selectivity, engendered by the 5′-*N*-methyl carboxamido moiety. The anti-oxidant functionality was incorporated at the N^6-position with the addition of the 5′-*N*-methyl carboxamide group and these were directly comparable to the previously tested novel adenosine A_1 receptor compound series (Gregg *et al.*, 2007). The receptor binding affinity of adenosine A_3 receptor agonists bearing a substituent known to give anti-oxidant activity in the N^6-position is reported in Table 2.2. All compounds showed low to sub-nanomolar K_i values to the high affinity agonist binding conformation of the adenosine A_3 receptor

with good selectivity over adenosine A_{2A} and A_{2B} receptor. However, adenosine A_1 receptor selectivity was observed generally only modestly, with adenosine A_3 receptor dominating. A functionalized linker was utilized to incorporate improvements in adenosine A_3 receptor affinity. The functionalized aniline linker series were highly potent with K_i values for adenosine A_3 receptor ranging from 2.25-45 nM. Compounds 7b (VCP439) is mildly selective for the adenosine A_3 receptor, whereas 7c (VCP438) was >100 fold selective versus adenosine A_1 receptor. Building on these substituted aniline structures, the incorporation of the "methylene spacer" in the benzylamino functionalised linkers produced 9a-c (VCP486, VCP485 and VCP487, respectively). All of these showed an elevated adenosine A_3 receptor selectivity (14-19 fold), and retained low nanomolar affinities (Devine *et al.,* 2010).

In conclusion, a simulated ischemia model was developed and characterized in a cardiac cell line. This model was used for the preliminary pharmacological screening of novel adenosine receptor selective compounds for their cardioprotective effect to select the effective compounds for further pharmacological evaluation. With the use of our SI model (12 hrs) in a cardiac cell line, we have demonstrated the cardioprotective effect of novel adenosine A_1 and A_3 agonists by reduced cell death. Development of simulated ischemia model through the evaluation of various time points of SI buffer exposure to cardiac cell line involved a thorough procedure of characterisation and validation to use as a tool for the preliminary pharmacological screening of the novel cardioprotective compounds. The adenosine A_1 compound VCP28 (2e) have shown the promising cardioprotective effect in this model at lower nanomolar concentration, which is further investigated at a range of

concentration in the same models. The effect of VCP28 on postischemic functional recovery during ischemia and reperfusion injury was investigated using Langendorff perfused isolated rat heart model, which is described in Chapter 5 in this thesis.

CHAPTER 3

Cardioprotection induced by adenosine A_1 receptor agonists in a cardiac cell ischemia model involves co-operative activation of adenosine A_{2A} and A_{2B} receptors by endogenous adenosine

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3.1. Abstract

Extracellular adenosine concentrations increase within the heart during ischemia and any exogenous adenosine receptor agonists therefore work in the context of significant local agonist concentrations. We evaluated interactions between A_1, A_{2A}, A_{2B} and A_3 receptors in the presence and absence of adenosine deaminase (ADA; to remove endogenous adenosine) in a cardiac cell ischemia model. Simulated ischemia (SI) was induced by incubating H9c2(2-1) cells in SI medium for 12 hrs in 100% N_2 gas prior to assessment of necrosis using propidium iodide (5μM) or apoptosis using AnnexinV-PE flowcytometry. N^6-Cyclopentyladenosine (CPA; 10^{-7}M) and N^6-(3-Iodobenzyl) adenosine-5'-N-methyluronamide (IB-MECA; 10^{-7}M) reduced the proportion of non-viable cells to 30.87 ± 2.49% and 35.18 ± 10.30% respectively (% of SI group). In the presence of ADA the protective effect of CPA was reduced (62.82 ± 3.52% non-viable), whilst the efficacy of IB-MECA was unchanged (35.81 ± 3.84% non-viable; $p<0.05$; n=3-5; SI vs. SI+ADA). The protective effects of CPA and IB-MECA were abrogated in the presence of their respective antagonists DPCPX and MRS1191, whilst A_{2A} and A_{2B} agonists had no significant effect. CPA-mediated protection was abrogated in the presence of both A_{2A} (ZM241385; 50 nM) and A_{2B} (MRS1754; 200 nM) antagonists (n=3-5, $p<0.05$). In the absence of endogenous adenosine significant protection was observed with CPA in presence of CGS21680 or LUF5834 ($p<0.05$ vs. SI + ADA + CPA). Apoptosis (14.35 ± 0.15 % of cells in SI + ADA group; $p<0.05$ vs. control) was not significantly reduced by CPA or IB-MECA. In conclusion, endogenous adenosine makes a significant contribution to A_1 agonist-mediated prevention of necrosis in this SI model by co-operative interactions

with both A_{2A} and A_{2B} receptors, but does not play a role in A_3 agonist-mediated protection.

3.2. Introduction

The significant role of adenosine receptor activation in protecting the myocardium from ischemic damage is well established. Exogenous adenosine (Lasley *et al.*, 1998; Peart *et al.*, 2002) and adenosine A_1 (Lasley *et al.,* 1990; Lasley *et al.,* 2007) and A_3 (Tracey *et al.,* 1997; Maddock *et al.,* 2002) receptor agonists consistently reduce cardiac infarct size and improve functional recovery in isolated heart models. A_{2A} receptor agonism has been observed to mediate protection (Maddock *et al.*, 2001) or have no effect under different experimental conditions (Peart *et al.*, 2002), and much of the A_{2A} mediated "postconditioning" protection is known to derive from inhibition of lymphocyte-mediated inflammation (Yang *et al.*, 2005). The role of A_{2B} receptors in ischemic protection has been difficult to distinguish from that of A_{2A} receptors prior to the recent availability of selective agonists and antagonists. However, a study involving a comparison of ischemic pre-conditioning in A_1, A_{2A}, A_{2B} and A_3 knockout mice indicated that A_{2B} receptors may play a pivotal role in this phenomenon (Eckle *et al.*, 2007). In cultured cardiomyocytes both A_1 and A_3 agonists have been shown to reduce necrotic cell death (Safran *et al.*, 2001), whilst A_2 receptor agonists have no protective effects in isolated cardiomyocytes (Bes *et al.*, 2002).

Under ischemic conditions, extracellular endogenous adenosine concentration increases (Van Wylen *et al.,* 1992; Headrick 1996) due to a variety of events that are up-regulated, including extracellular cAMP conversion to adenosine by ecto-5'-nueclotidase (Jackson *et al.*, 2007). Thus, exogenous adenosine receptor agonists exert their effects in the context of significant levels of the endogenous ligand (Headrick *et al.*, 2003).

A number of studies have provided evidence of interactions between adenosine receptors, including A_1, A_{2A} and A_{2B} in rat heart (Lopes *et al.,* 1999; Norton *et al.,* 1999; Germack *et al.,* 2005; Tikh *et al.,* 2006). Lopes et al (1999) found that in rat hippocampal and cortical nerve terminals, the A_{2A} receptor agonist CGS21680 reduced the binding affinity of the A_1 adenosine receptor agonist CPA, an effect blocked by the A_{2A} receptor antagonist ZM241385. The A_1 receptor-mediated anti-adrenergic effect in isolated mouse heart has been shown to be inhibited by A_{2A} receptor activation (Tikh *et al.*, 2006).

There are few reports of interactions between A_3 adenosine receptors and other adenosine receptors. A_1 and A_3-mediated cardioprotective effects appear to occur independently; Stambaugh et al (1997) reported that A_1 and A_3 receptor activation reduces ischemic damage in chick cardiac ventricular myocytes after prolonged hypoxia, and MRS1191 did not reduce the protective effect of the A_1 agonist CCPA. There is some evidence that A_{2A} receptor activation plays a role in effects produced by A_3 adenosine receptor agonists. Maddock and colleagues reported that an A_{2A} receptor antagonist reversed the protective effects of the A_3 agonist Cl-IB-MECA during reperfusion in isolated rat heart, although these authors suggested that this may be related to complex pro-apoptotic and anti-apoptotic effects of A_3 receptor activation (Maddock *et al.*, 2002).

Recently Lasley et al reported that antagonism of A_2 receptors attenuated the protective effect of adenosine A_1 agonists in a rat *in-vivo* heart preparation (Lasley *et al.*, 2007). The implication from this study was that activation of A_2 receptors by endogenous adenosine

plays a role in adenosine A_1 receptor-mediated cardioprotection. We therefore sought to determine whether the removal of endogenous adenosine using adenosine deaminase would (a) reduce the cardioprotection provided by A_1 or A_3 agonists, and (b) negate the role of A_2 receptors in A_1-mediated cardioprotection. We therefore investigated the interactions between A_1, A_{2A}, A_{2B} and A_3 receptor activation in a model of ischemia using H9c2(2-1) cardiac cells, in the presence and absence of endogenous adenosine.

3.3. Materials and Methods

3.3.1. Cell Culture and Simulated Ischemia

The H9c2(2-1) embryonic rat atrial cell line (American Type Culture Collection-ATCC, Manassas, VA, USA) was used for this study. The cell line was grown in Dulbecco's modified Eagle's medium containing 4 mM L-glutamate, 4.5 g/L glucose, 3.7 g/L sodium bicarbonate, 100 U/ml penicillin and 100 mg /ml streptomycin supplemented with 10% fetal bovine serum (Invitrogen, Mount Waverley, VIC, Australia) in a 5% CO_2 incubator. Cells were used at 60-70 % confluence and plated at the density of $1x10^6$ cells/ml in a 96-well flat-bottomed plate one day prior to assay. Simulated ischemia was induced using conditions developed in our lab and described previously (Gregg *et al.*, 2007). In short, ischemia was achieved by incubating the cells in hypoxic simulated ischemia (SI) medium at pH 6.4 containing (in mM): 137 NaCl, 3.5 KCl, 0.88 $CaCl_2.2H_2O$, 0.51 $MgSO_4.7H_2O$, 5.55 D-glucose, 4 HEPES, 10 2-deoxy-D-glucose and 20 DL-lactic acid (Sigma, Castle Hill, NSW, Australia) plus 2% fetal bovine serum. Cells were incubated under nitrogen (100% N_2 gas atmosphere) for 12 hrs at 37^oC. The ischemia model induced both apoptosis and necrosis in cultured H9c2(2-1) cells. Under these conditions approximately 40% of all cells stained positive to propidium iodide (which was normalized to 100% SI) indicating necrosis and 10.05 ± 0.85% cells were AnnexinV-PE and 7-Amino-actinomycin (7-AAD) positive cells were indicating end stage apoptosis. Fresh simulated ischemia medium was prepared for each experiment. The control group of cells was kept in a normal oxygen incubator (5% CO_2 atmosphere) for the same period of time. In the control group HEPES buffer without 2-deoxy-D-glucose and DL-lactic acid was used.

3.3.2. General Experimental Protocol

H9c2(2-1) cells were incubated in either normal medium (controls) or hypoxic SI medium. Agonists were diluted freshly with PBS to the appropriate concentrations from stock solutions and added at the start of simulated ischemia. When antagonists were used, they were added 20 min before addition of the agonist. Adenosine deaminase; 1U/ml (Maggirwar *et al.*, 1994) when used to remove endogenously released adenosine (convert endogenous adenosine to inosine) from left atria, papillary muscles and H9c2(2-1) cells during simulated ischemia, was added before addition of either agonists or antagonists. The experimental protocol is summarized in Figure 3.1.

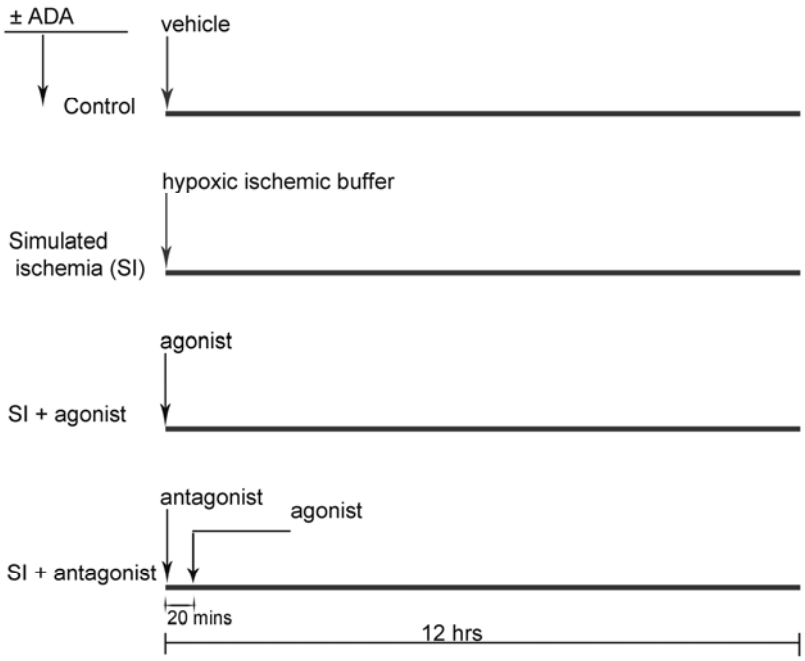

Figure 3.1. Schematic diagram showing the timing of simulated ischemia and drug addition in different groups in the absence and presence of ADA (adenosine deaminase). Cells were exposed to simulated ischemic conditions for 12 hrs with agonists and antagonists.

3.3.3. Experimental Details

Experimental protocols are described below for each of four studies (A-E);

(A) Effect of adenosine A_1, A_{2A}, A_{2B} and A_3 receptor activation during ischemic conditioning of H9c2(2-1) cells- (i) control, (ii) simulated ischemia (SI), (iii) SI + combined adenosine receptor antagonists contained either DPCPX, ZM241385, MRS1754 or MRS1191 (10^{-6}M; selective A_1, A_{2A}, A_{2B} and A_3 antagonists respectively), (iv) SI + ADA, (v) SI + agonists-one of the following CPA, CGS21680, LUF5834 or IB-MECA (10^{-8} M; selective A_1, A_{2A}, A_{2B} and A_3 agonists respectively).

(B) Effect of A_1 and A_3 receptor activation in the presence and absence of endogenous adenosine during simulated ischemia- (i) SI + CPA (10^{-12} – 10^{-6} M) ± DPCPX (10^{-6} M), (ii) SI + CPA + ADA ± DPCPX, (iii) SI + IB-MECA (10^{-12} – 10^{-6} M) ± MRS1191 (10^{-6} M), (iv) SI + IB-MECA + ADA ± MRS1191.

(C) Effect of A_{2A}, A_{2B} and A_3 antagonist on A_1 receptor-mediated protection against ischemia- (i) SI + CPA + ZM241385 (50 nM), (ii) SI + CPA + MRS1754 (200 nM), (iii) SI + CPA + MRS1191 (10^{-6} M).

(D) Effect of A_1, A_{2A} and A_{2B} antagonist on A_3 receptor-mediated protection against ischemia- (i) SI + IB-MECA + DPCPX (10^{-6}M), (ii) SI + IB-MECA + ZM241385 (50 nM), (iii) SI + IB-MECA + MRS1754 (200 nM). The concentration of agonists was selected on the basis of their EC_{50} values obtained from their concentration-response curves.

(E) Effect of A_1 agonist combined with A_{2A} and A_{2B} agonist in the absence of endogenous adenosine against ischemia- (i) SI + ADA + CPA (10^{-8}-10^{-7}M) + CGS21680 (10^{-8}-10^{-7}M) ± ZM241385 (50nM), (ii) SI + ADA + CPA (10^{-8}-10^{-7}M) + LUF5834 (10^{-8}-10^{-7}M) ± MRS1754 (200nM).

3.3.4. Cell viability (Propidium Iodide) Assay and Imaging of H9c2(2-1) cells

For detection of non-viable cells (necrosis) in all groups**,** a propidium iodide assay was used. At the end of the period of simulated ischemia, 5μM propidium iodide (PI; Sigma, NSW, Australia) was added to each well and incubated for 15 min in the dark. After 15 min images were taken using an inverted fluorescence microscope connected to a SPOT RT camera (Nikon Eclipse TE2000U, Nikon Instruments, Japan) at 535 nm excitation wavelength achieved by DG-4 light box (Shutter Instruments, USA) and 617 nm emission filters. Duplicate wells were used for each experiment, and each experiment was repeated three times. From each well 4 images were taken, and propidium iodide positive cells were quantified with using Scion Image Alpha 4.0.3.2 software (NIH Image, National Institute of Health, USA) then normalized to the SI treatment group (i.e. necrosis was expressed as % cell death with respect to the control SI group using this model).

3.3.5. Apoptosis (AnnexinV Flow-Cytometry) Assay

AnnexinV is Ca^{2+} dependent phospholipid-binding protein with high affinity for cell membrane phosphatidylserine linkage (conjugated with fluorochrome phycoerythrin; PE) was used in conjunction with a vital dye 7-Amino-actinomycin (7-AAD) to measure viable cells (AnnexinV-PE and 7-AAD negative), early apoptosis (AnnexinV-PE positive, 7-AAD

negative), end stage apoptosis and death (AnnexinV-PE and 7-AAD positive) (Vermes *et al.*, 1995). AnnexinV-PE assays were performed on cells treated as described for protocols A and B. H9c2(2-1) cells were incubated in 24-well plates at a density of $1x10^6$ cells/ml in cell growth medium as described previously. Simulated ischemia was produced as described above. After 12 hrs of hypoxic SI the cells were trypsinised and removed from the plate. An AnnexinV-PE apoptosis detection kit I (BD Biosciences- Pharminogen, San Diego, California, USA) was used to assess the incidence of apoptosis. AnnexinV-PE and 7-AAD staining was performed as per the manufacturer's standard protocol. The cells were then analyzed within one hour of staining using a BD FACS Canto II system (BD Biosciences, Immunocytometry systems, California, USA). Data were analyzed using FACSDiva software v 5.0.1 (BD Biosciences, Immunocytometry systems, California, USA).

3.3.6. Drugs and Preparation of Stock Solutions

CPA, IB-MECA, DPCPX, MRS1754, MRS1191 and adenosine deaminase (adenosine deaminase from bovine spleen Type X, buffered aqueous glycerol solution, 130 units/mg protein) were purchased from Sigma-Aldrich (Castle Hills, NSW, Australia). CGS21680, ZM241385 was purchased from Tocris Biosciences (Ellisville, Missouri, USA). All other reagents and chemicals were purchased from Sigma-Aldrich (Castle Hills, NSW, Australia). LUF5834 is generously provided as a gift sample by Dr Margot W. Beukers and Dr Thea Mulder-Krieger, Division of Medicinal Chemistry, Leiden/Amsterdam Center for Drug Research (LACDR), Gorlaeus Laboratories, Leiden University, 2300 RA Leiden, The Netherlands. All agonists and antagonists were dissolved in dimethyl sylfoxide (DMSO)

and diluted to make 10^{-6}M stock solutions containing no more than 0.1% DMSO and stored at -20^{0}C. At the time of experiment stock solutions were freshly diluted in PBS.

3.3.7. Statistical Analysis

Data were expressed as mean ± SEM in all cases. Cell viability data were analyzed by one way ANOVA using Bonferroni's post-hoc test for multiple comparisons. For the cell viability assay non-viable cells from SI group were normalized to 100%. The effects of adenosine receptor agonists and antagonists in absence and presence of ADA, were analyzed by two way ANOVA followed by Bonferroni's post-hoc tests. A P-value of 0.05 was used to indicate significant difference.

3.4. Results

3.4.1. Simulated ischemia in the presence and absence of endogenous adenosine and A_1, A_{2A}, A_{2B} and A_3 antagonists

Incubation of cells in HEPES buffer for 12 hrs resulted in a low percentage of non-viable (propidium iodide positive) cells (Figure. 3.2a). Simulated ischemic conditions resulted in a significant increase in the number of non-viable cells (48.42 ± 8.11% cells were propidium iodide positive, $p<0.05$ vs. control; Figure. 3.2b). The SI group was assigned a value of 100%; therefore other treatments were expressed as % normalized to the SI group. The adenosine A_1 receptor antagonist- DPCPX, the A_{2A} antagonist ZM241385, the A_{2B} antagonist MRS1754 and the A_3 antagonist MRS1191 (10^{-6}M) individually did not increase the proportion of non-viable cells (100.9 ± 8.46%, 97.64 ± 6.04%, 112.3 ± 12.65% and 107.6 ± 16.86% respectively with reference to the SI group). However, the combination of all four adenosine receptor antagonists significantly increased the number of non-viable cells (133.2 ± 8.28% with reference to the SI group, $p<0.05$ vs. SI; Figure. 3.2d, e). To investigate the role of endogenous adenosine in protection of cells from ischemia, cells were incubated with SI medium in the absence and presence of adenosine deaminase (ADA, 1U/ml) for 12 hrs. Eradication of endogenous adenosine by treatment with ADA significantly increased the proportion of non- viable cells to 125.4 ± 7.76% with reference to the SI group ($p<0.05$ vs. SI, Figure. 3.2c).

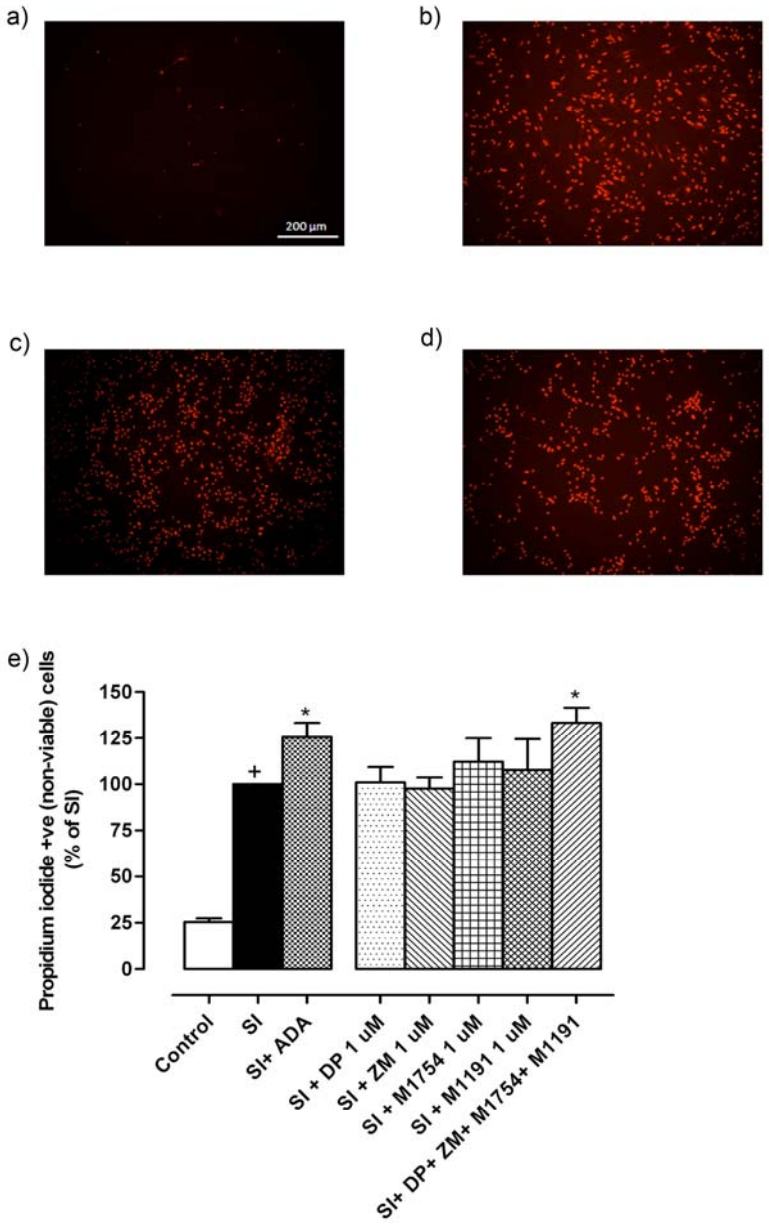

Figure 3.2. Characterization of the simulated ischemia (SI) model using H9c2(2-1) cells. Cells were grown for 12 hrs either in normal medium (control) or hypoxic simulated ischemia medium alone or with adenosine deaminase (ADA) with A_1, A_{2A}, A_{2B} and A_3 antagonist treatments. Propidium iodide (5 µM) nuclear staining was used to determine non-viable cells. The simulated ischemia data was normalized to 100% (mean ± SEM, n= 3-5). $^{+}P < 0.05$ vs. control, $^{*}P < 0.05$ vs. SI. Representative photomicrographs of **(a)**

control, **(b)** SI, **(c)** SI + ADA and **(d)** SI + A_1, A_{2A}, A_{2B} and A_3 antagonists. **(e)** Histogram showing after 12 hrs of ischemia H9c2(2-1) nuclei of necrotic or non-viable cells (10x magnification) were readily imaged (DP = DPCPX- A_1 antagonist; ZM = ZM241385- A_{2A} antagonist; M1754 = MRS1754- A_{2B} antagonist; M1191 = MRS1191- A_3 antagonist).

3.4.2. The effect of A_1, A_{2A}, A_{2B} and A_3 receptor activation during simulated ischemia

As shown in fig. 3.3a, CPA (A_1 agonist) and IB-MECA (A_3 agonist) induced significant decreases in the number of non-viable cells during simulated ischemia to 33.07 ± 3.16% and 42.54 ± 10.89% respectively with reference to the SI group (10^{-8}M; $p<0.05$ vs. SI), whilst CGS21680 (A_{2A} agonist) and LUF5834 (A_{2B} agonist) alone had no effect.

3.4.3. Effects of A_1 and A_3 receptor activation during simulated ischemia in the absence and presence of endogenous adenosine

When cells were incubated with CPA, ischemic protection was observed in a concentration dependent manner in the absence and presence of ADA. Fig 3.3b and 3.3c illustrate the concentration response curves for CPA (10^{-13} to 10^{-6} M) in the absence and presence of ADA. CPA (10^{-7}M) caused a significant protective effect (30.87 ± 2.49% of non-viable cells with reference to the SI group, $p<0.05$ CPA vs. CPA + ADA) in the absence of ADA. In the presence of ADA, however, the protective effect of CPA (10^{-7}M) was reduced (62.82 ± 3.52% of non-viable cells with reference to the SI group). Both in the absence and presence of ADA, the effect of CPA was reversed significantly by DPCPX ($p<0.05$ vs. CPA or CPA + ADA). Figure. 3.3d and 3.3e illustrate that, unlike CPA, the presence of ADA had little effect on the concentration-response curves for IB-MECA. In the absence

and presence of ADA, IB-MECA (10^{-7}M) produced a significant protective effect (35.18 ± 10.30% and 35.81 ± 3.84% of non-viable cells with reference to the SI group respectively). In absence and presence of ADA IB-MECA-mediated ischemic protection was significantly reversed by MRS1191 ($p<0.05$ vs. IB-MECA or IB-MECA + ADA).

There was no significant difference between the maximum protection produced by CPA and that of IB-MECA in the absence of ADA (30.87 ± 2.49% and 35.18 ± 10.30% of non-viable cells with reference to the SI group respectively at 10^{-7}M). However, in the presence of ADA, the maximum protective effect of IB-MECA (35.81 ± 3.84% of non-viable cells with reference to the SI group, 10^{-7}M) was greater than the maximum protective effect of CPA (62.82 ± 3.52%; 10^{-7}M).

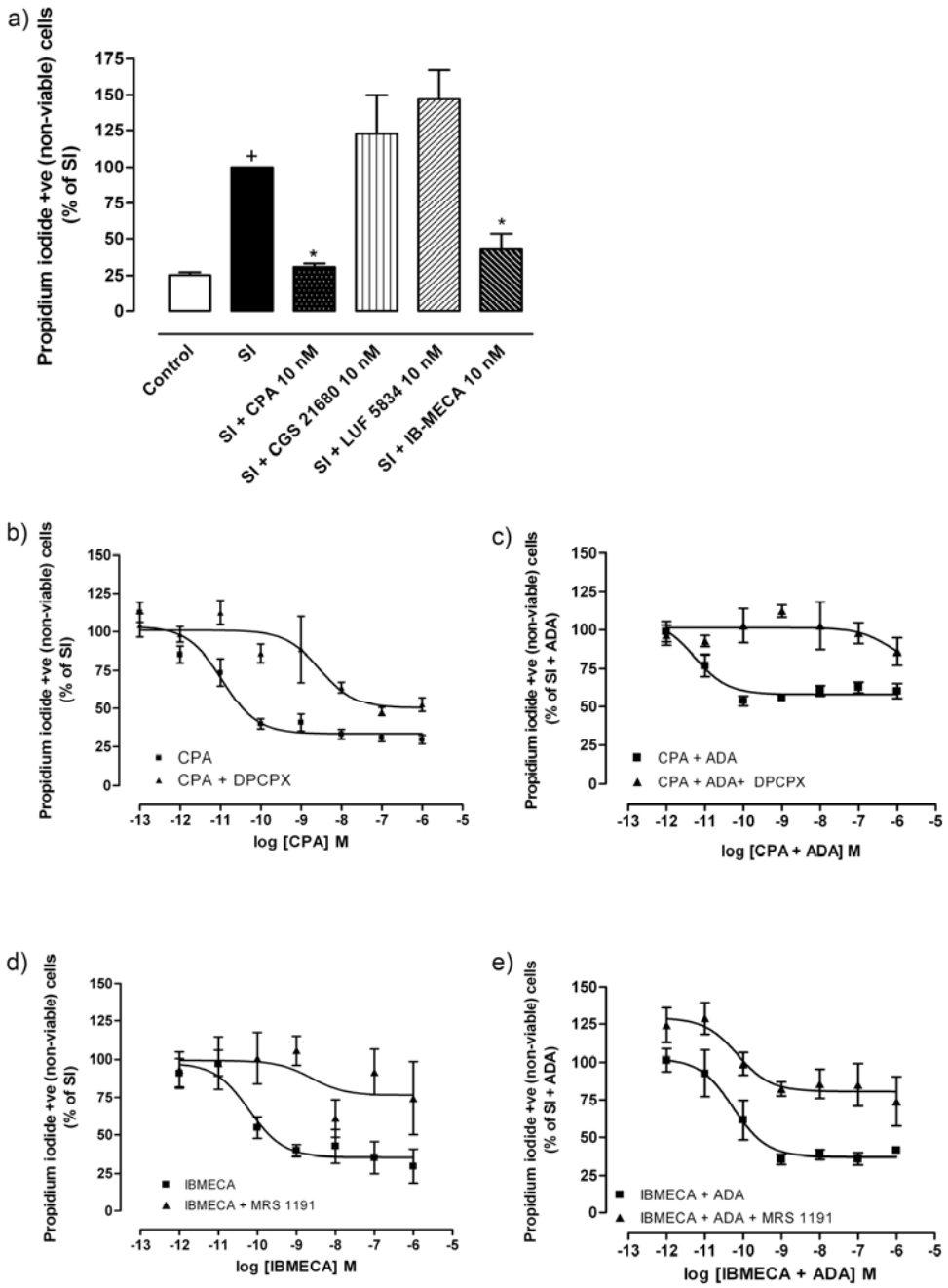

Figure 3.3. Effect of A_1, A_{2A}, A_{2B} and A_3 receptor activation on cell survival during simulated ischemia. **(a)** Cells were grown in normal medium, hypoxic SI medium alone or with CPA- adenosine A_1 receptor agonist, CGS21680- adenosine A_{2A} receptor agonist, LUF5834- adenosine A_{2B} receptor agonist or IB-MECA- adenosine A_3 receptor agonist (10^{-}

^{8} M). Concentration response curves for CPA- A_1 agonist **(b, c)** and IB-MECA- A_3 agonist **(d, e)** with their antagonists DPCPX and MRS 1191 respectively in presence and absence of adenosine deaminase (ADA). Cells were stained with propidium iodide (5 μM) to determine non-viable cells. (mean ± SEM, n= 3-5) $^{+}$P <0.05 vs. control group, *P <0.05 vs. drugs treated group.

3.4.4. Apoptosis (AnnexinV-PE Flow-Cytometry) Assay

A significant increase in the number of end stage apoptotic cells (AnnexinV-PE and 7-AAD positive) was observed when cells were incubated in SI medium in the presence of ADA (10.05 ± 0.85%, p<0.05 vs. control; Figure. 3.4). However, early stage apoptotic cell population (AnnexinV-PE positive, 7-AAD negative) was not changed significantly in all groups (P.0.05 vs. control). The slight decrease in end stage apoptotic cell population (AnnexinV-PE and 7-AAD positive cells) observed in CPA and IB-MECA-treated cells in the presence of ADA was not significant (Figure. 3.4f, P>0.05 vs. SI + ADA).

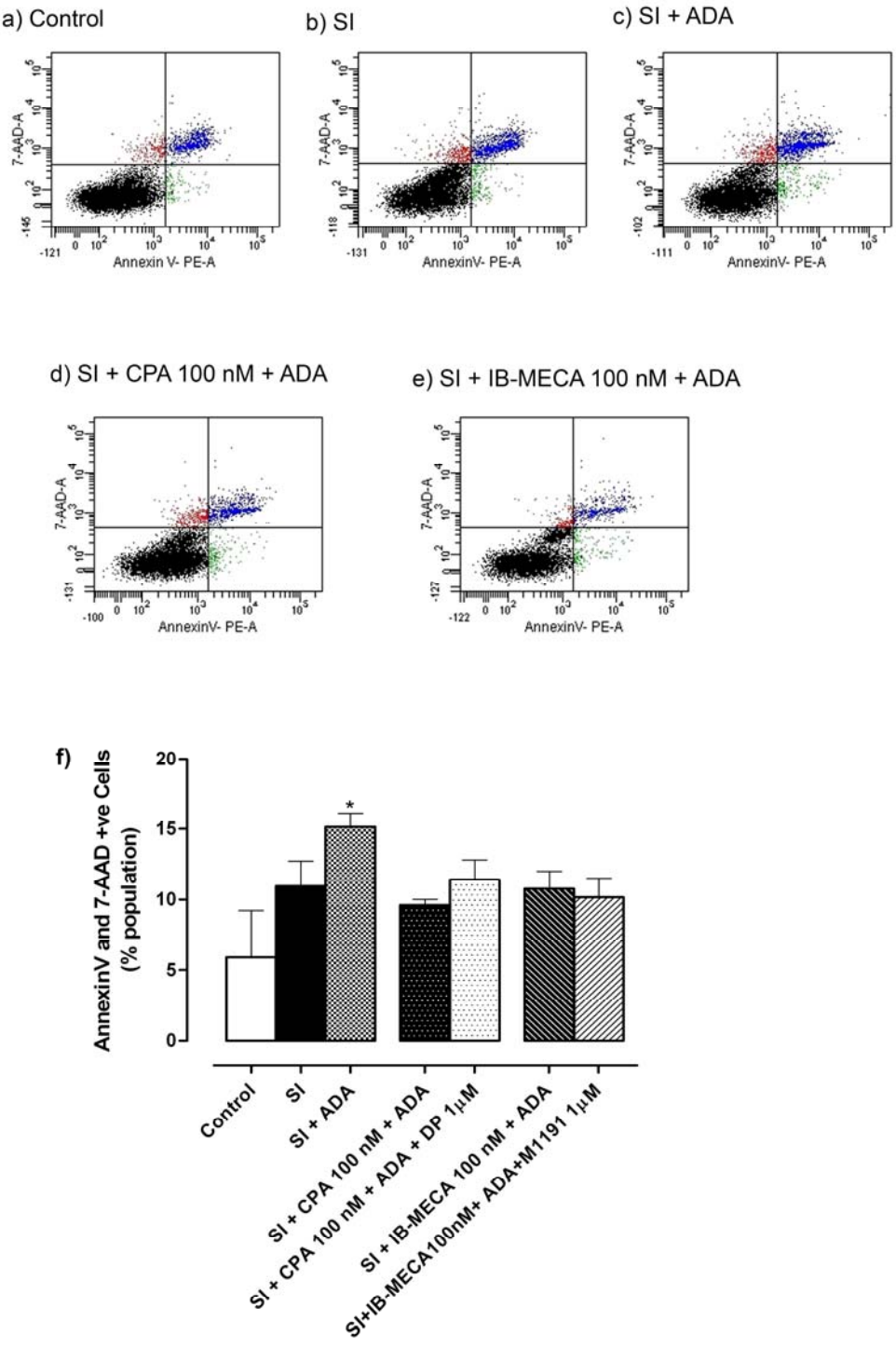

Figure 3.4. Dot plot of AnnexinV-PE /7-AAD flow cytometry of H9c2(2-1) cells after 12 hrs of simulated ischemia **(a-e)**. The lower left quadrant in each panel represents viable

cells (AnnexinV-PE and 7-AAD negative). The lower right quadrants represent early stage apoptosis (AnnexinV-PE positive, 7-AAD negative). The upper right quadrants represents end stage apoptosis (AnnexinV-PE and 7-AAD positive). Representative histogram of treatment groups **(f)** shows percent mean ± SEM of end stage apoptotic cell population in presence of ADA. (n= 3-5) $^{*}P < 0.05$ vs. Control group.

3.4.5. Effect of A_{2A}, A_{2B} and A_3 receptor antagonists on A_1 receptor-mediated protection against ischemia

In the absence of ADA – i.e. in the presence of endogenous adenosine - CPA-mediated protection (27.32 ± 3.26% of non-viable cells with reference to the SI group, 10^{-7}M), was reversed by both 50 nM ZM241385 and 200 nM MRS1754 (100.8 ± 10.30% and 209.9 ± 30.95% respectively of non-viable cells with reference to the SI group, 10^{-7}M, $p<0.05$ vs. CPA, Figure. 3.5a). However, CPA-mediated protection was not significantly altered in the presence of MRS1191 (10^{-6}M, Figure. 3.5c). In presence of ADA, CPA-mediated protection (57.62 ± 4.86% of non-viable cells with reference to the SI group, 10^{-7}M) was not significantly reversed by 50 nM ZM241385 or 200 nM MRS1754 (68.19 ± 6.95% and 69.43 ± 8.37% of non-viable cells with reference to the SI group respectively, Figure. 3.5b).

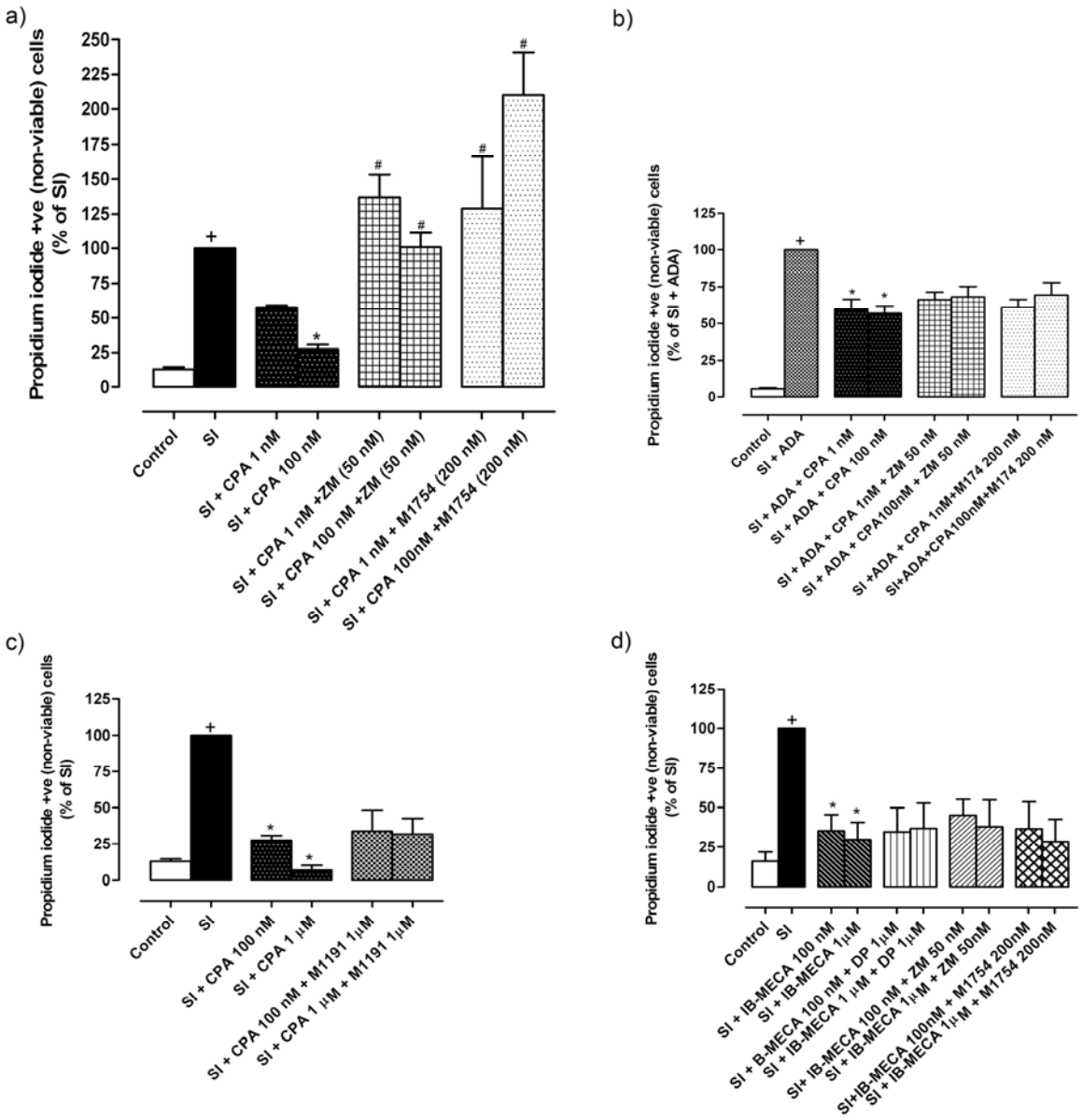

Figure 3.5. Effect of A_{2A}, A_{2B} and A_3 receptor antagonists on A_1 receptor-mediated protection against ischemia **(a)** In absence of ADA effect of A_{2A} and A_{2B} antagonists on A_1 receptor agonist, **(b)** In presence of ADA effect of A_{2A} and A_{2B} antagonists on A_1 receptor agonist, **(c)** Effect of A_3 antagonist on A_1 receptor agonist and **(d)** Effect of A_1, A_{2A} and A_{2B} antagonist on A_3 receptor agonist mediated cytoprotection against simulated ischemia**.** All drugs were added at the start of ischemia while the respective antagonists were added

20 min before agonist addition. (mean ± SEM, n= 3-5) $^{+}$P <0.05 vs. control group, *P <0.05 vs. SI + ADA and SI + ADA + CPA, $^{\#}$ P <0.05 vs. CPA treated groups (DP = DPCPX- A_1 antagonist; ZM = ZM241385- A_{2A} antagonist; M1754 = MRS1754- A_{2B} antagonist; M1191 = MRS1191- A_3 antagonist).

3.4.6. Effect of A_1, A_{2A} and A_{2B} receptor antagonists on A_3 receptor mediated protection against ischemia

The cardioprotective effect of IB-MECA (10^{-7}M) was not significantly altered in the presence of DPCPX (10^{-6}M), ZM241385 (50 nM) or MRS1754 (200 nM) (35.19 ± 10.30%, 34.58 ± 15.25%, 45.11 ± 9.98% 36.52 ± 17.0% respectively of non-viable cells with reference to the SI group; Figure. 3.5d).

3.4.7. Effect of A_1 agonist combined with A_{2A} and A_{2B} agonist in the absence of endogenous adenosine against ischemia

In the absence of endogenous adenosine CPA (10^{-8}M)-mediated protection increased significantly when incubated with 10^{-7}M CGS21680 or LUF5834 (30.87 ± 3.95 and 32.58 ± 4.55% respectively of non-viable cells with reference to the SI group). The protective effect of CGS21680 and LUF5834 when added in combination with CPA were reversed by the antagonists ZM241385 (50nM) and MRS1574 (200nM) respectively (Figure 3.6a and 3.6b).

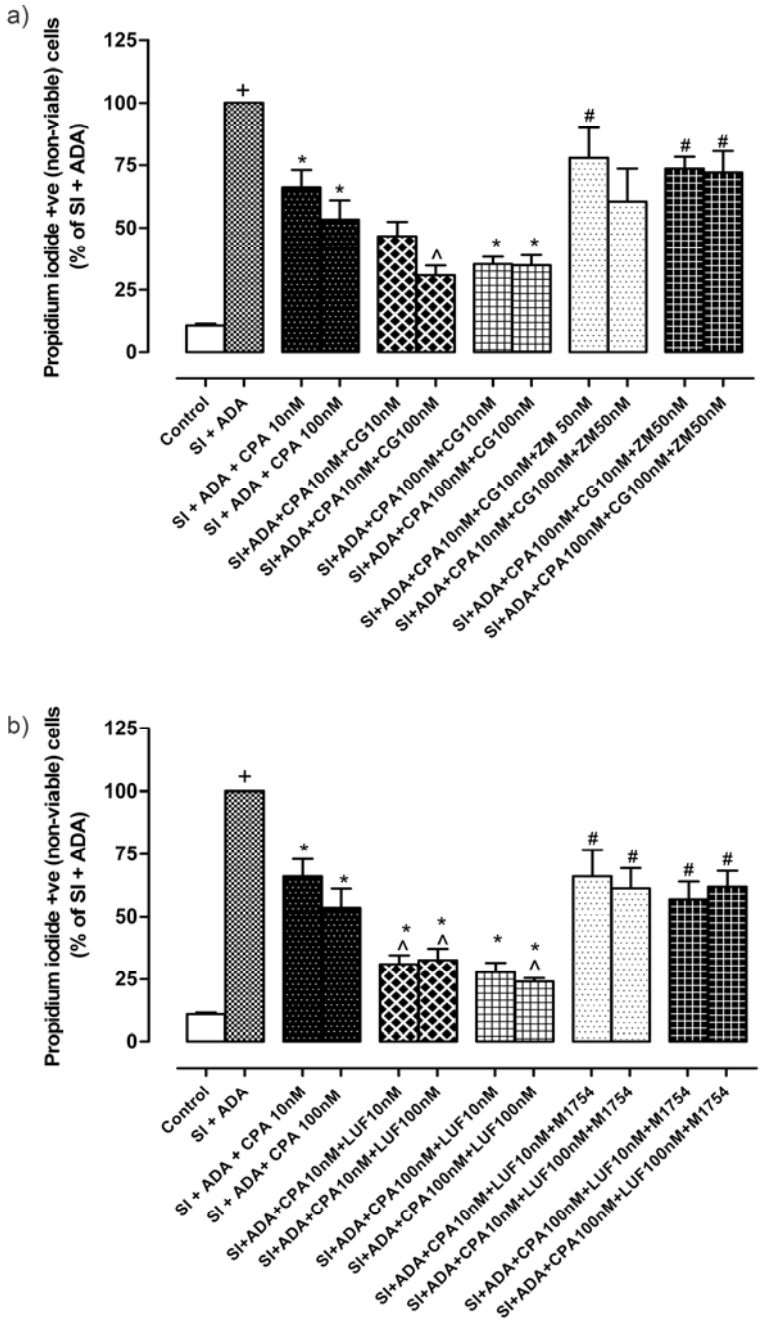

Figure 3.6. Effect of A_1 receptor agonist combined with A_{2A} or A_{2B} receptor agonist in the absence of endogenous adenosine against ischemia. (a) effect of A_1 receptor agonist alone and in the presence of A_{2A} receptor agonist; (b) effect of A_1 receptor agonist alone and in the presence of A_{2B} receptor agonist (mean ± SEM, n= 4). $^{+}P < 0.05$ vs. control group, $^{*}P$

<0.05 vs. SI + ADA treated group, ^P <0.05 vs. SI + ADA + CPA treated group, #P <0.05 vs. SI + ADA + CPA ± CGS21680 ± LUF5834 treated groups (CG = CGS21680- A_{2A} agonist; LUF = LUF5834- A_{2B} agonist; DP = DPCPX- A_1 antagonist; ZM = ZM241385- A_{2A} antagonist; M1754 = MRS1754- A_{2B} antagonist).

3.5. Discussion

A recent study by Lasley et al (2007) reported that the cardioprotective effects of the A_1 adenosine receptor agonist 2-chloro–N^6-cyclopentyladenosine (CCPA) were reversed in the presence of the A_2 receptor antagonist ZM241385 in isolated rat hearts. The results of the present study show that endogenous adenosine-mediated activation of both A_{2A} and A_{2B} receptors is required for the prototype A_1 agonist CPA-mediated inhibition of necrotic cell death in a cardiac cell model of ischemia. We observed that endogenous adenosine had a small protective effect via synergy between adenosine A_1, A_{2A}, A_{2B} and A_3 receptors and that removal of endogenous adenosine significantly inhibited such A_1-mediated cardioprotection but not A_3-mediated cardioprotection. In common with other research groups we observed a profound effect of exogenous agonists in the context of a model in which the levels of extracellular endogenous adenosine are elevated (Casati *et al.,* 1997; Stambaugh *et al.,* 1997; Merkel *et al.,* 1998; Gregg *et al.,* 2007)**.**

We titrated the time of ischemia against necrotic cell death, and chose a time of ischemia (12 hours) that resulted in significant (48.42 ± 8.11 % of total cells) but not catastrophic cell death, so as to be able to observe both protection, and worsening, of ischemic cell death. In the presence of ADA, we observed an increased percentage of necrotic cells compared to the simulated ischemia alone treatment group (67.28 ± 11.42 % with reference to the SI group staining positively for propidium iodide) suggesting that increased (extracellular) endogenous adenosine after ischemic damage in the cells was responsible for protection from necrotic cell death. This hypothesis is supported by the demonstration that ADA deficiency in normoxic and ischemic mouse heart increases ischemic tolerance and

also A_1 and A_2 receptor involvement in protection (Willems *et al.*, 2006). Endogenous adenosine is released during ischemia, at least in part via the extracellular cAMP-adenosine pathway (Jackson *et al.*, 2007) and significantly increased adenosine levels have been found in interstitial and intravascular fluid (Van Wylen 1994; Kin *et al.,* 2005b). Ischemia increases interstitial adenosine concentrations to approximately 2 μM in mice (and higher levels in rats) and this elevated adenosine activates adenosine A_1 receptor subtypes (Peart *et al.*, 2000), which protects against ischemic injury (Toombs *et al.,* 1992; Strickler *et al.,* 1996). Thus the role of endogenous adenosine in this study is relevant to the *in vivo* setting. We found that the individual addition of A_1, A_{2A}, A_{2B} or A_3 receptor antagonists had no effect on the proportion of necrotic cells, but that the combination of all four receptor antagonists did significantly increase the proportion of necrotic cells to a level equivalent to that in the SI + ADA treatment group. Thus, we conclude that adenosine works by additive or synergistic interactions between adenosine A_1, A_{2A}, A_{2B} and A_3 receptors in this model of ischemia to limit necrotic cell death.

As expected, we observed CPA-mediated cardioprotection when added at the onset of ischemia, consistent with previous studies using isolated rat and mouse heart (Vinten-Johansen *et al.,* 1999; Peart *et al.,* 2000; Linden 2001; Headrick *et al.,* 2003). In the present and our previous study (Gregg *et al.*, 2007) we observed significant ischemic protection with low (10^{-8}M) concentrations of CPA, which suggest that increased extracellular endogenous adenosine during ischemia, may mimic the exogenous A_1 agonist (CPA) effect against ischemic protection. Ischemic protection was also observed in our study when IB-MECA was administered at the onset of ischemia. This is consistent with *ex vivo* studies in

isolated rat heart (Maddock *et al.*, 2002) isolated rabbit heart (Jordan *et al.*, 1999; Tracey *et al.*, 2003), *in vivo* open chest anesthetised dog model (Auchampach *et al.*, 2003) and rabbit heart (Tracey *et al.*, 2003). We observed no protection with A_{2A} (CGS21680) and A_{2B} (LUF5834) (Beukers *et al.*, 2004) agonists alone when they were added at the onset of ischemia. These observations are supported by a study in which the rat isolated heart treated with CGS21680 (10^{-6}M) at the onset of ischemia or reperfusion showed no functional recovery (Finegan *et al.*, 1996).

In the presence of endogenous adenosine CPA showed greater maximal ischemic protection (maximal protection ~70% of SI) than in SI + ADA treated cells (maximal protection ~40% of SI). However, IB-MECA induced a similar ischemic protection effect in the presence and absence of endogenous adenosine (maximal protection ~ 35% of SI). The protection induced by both CPA and IB-MECA was reversed by their respective selective antagonists DPCPX and MRS1191. The possibility exists that CPA is exerting protective effects via acting on adenosine receptors other than the A_1 receptor. This is not likely to be the case given that the maximum protective effect of CPA was observed at around 10^{-9}M, and that CPA is 30-8000-fold selective for A_1 vs. either A_{2A}, A_{2B} or A_3 adenosine receptors (K_i value 2.3nM at A_1 vs. 794nM, 18600nM, and 72nM at A_{2A}, A_{2B} or A_3 adenosine receptors respectively) (Jacobson *et al.*, 2006).

In addition to necrotic cell death we also measured apoptotic cell death. AnnexinV-PE serves as a marker for membrane alteration during the ischemia and binds to phospholipid phosphatidylserine which is translocated during the apoptosis (Vermes *et al.,* 1995;

Narayan *et al.*, 2001). In our model there was a low level of apoptosis in the control group, and no significant increase in apoptosis observed in the SI group. In the presence of ADA, a significant increase in end stage apoptosis (AnnexinV-PE positive and 7-AAD positive) was observed. We found that CPA and IB-MECA had no significant effect on the end stage apoptosis in the SI + ADA treatment group. A_1 and A_3 receptor agonists (CCPA, IB-MECA respectively; 10^{-7}nM) have previously been shown to mediate simulated preconditioning protection in the cultured chick ventricular myocyte model after hypoxia (Strickler *et al.*, 1996). Taken together, these findings suggest CPA and IB-MECA can prevent apoptosis in conditions where endogenous adenosine-mediated activation of adenosine receptors combines with the A_1 and A_3 agonism respectively. In our model, however in the presence of endogenous adenosine, the percent of end stage apoptotic cells in the SI treatment group was low, and therefore we did not observe a significant reduction in apoptosis when CPA or IB-MECA was present. Therefore we looked for the interaction between A_1, A_{2A}, A_{2B} and A_3 receptors by assessing necrotic cell death in our SI model.

To assess the contribution of endogenous adenosine to the protective effects observed when an A_1 or A_3 receptor agonists was introduced in the SI model, ZM241385 (A_{2A} antagonist), MRS1754 (A_{2B} antagonist) and MRS1191 (A_3 antagonist) were used. It is improbable that ZM241385 (K_i value for binding at the human A_{2A} receptor 1.6 nM, vs. 774 nM at A_1) (Jacobson *et al.*, 2006) or MRS1754 (1.97 nM at A_{2B} vs. 403 nM at A_1) (Jacobson *et al.*, 2006) directly antagonised A_1 receptors. We observed that ZM241385 and MRS1754 reversed CPA-mediated ischemic protection in the presence of endogenous adenosine but not when ADA was employed. These data indicate that endogenous adenosine mediated

activation of both A_{2A} and A_{2B} receptors contribute to the protective effect observed when CPA is added in this SI model. In IB-MECA treated groups cardioprotection was not reduced by DPCPX, ZM241385 and MRS1754, showing that A_3 mediated ischemic protection is independent of other adenosine receptor activation by endogenous adenosine.

The result of the present study should be interpreted with a caveat that the adenosine A_1 and A_{2A} antagonist DPCPX and ZM241385 respectively, have a relatively high binding affinity for the adenosine A_{2B} receptor as well in various rodent species, which has been reported recently (Auchampach *et al.,* 2009). The adenosine A_{2B} antagonist MRS1754 (200 nM) used in the present study has shown some binding affinity for the adenosine A_1 receptor in rat (Table 1.2 B). In hindsight, a lower concentration of DPCPX and MRS1754 would have been appropriate.

In the absence of endogenous adenosine, addition of either exogenous adenosine A_{2A} or A_{2B} receptor agonist in combination with the adenosine A_1 receptor agonist (CPA) significantly increased ischemic protection compare to CPA alone. These finding suggest that activation of either adenosine A_2 receptor is sufficient to augment the protective effect of the adenosine A_1 receptor agonist.

Thus, we confirm in cultured cardiac cells the findings of Lasley et al (2007) that simultaneous activation of A_2 receptors is required for full protection using exogenous A_1 adenosine receptor agonists in isolated hearts. We further show that either of A_{2A} or A_{2B} receptor activation is sufficient to produce "co-operative A_1 and A_2 ischemic protection".

Low concentrations of the A_1 agonist CPA are protective in the presence, but not the absence of endogenous adenosine. The addition of both adenosine A_{2A} and A_{2B} receptor agonist (CGS21680 and LUF5834; 10^{-8}-10^{-7}M) did not produce a greater effect then either agonist alone (data not shown).

Although A_1 and A_2 receptors have opposing effects on adenylyl cyclase, adenosine A_1, A_{2A} and A_{2B} receptor activation results in activation of a common signalling pathway, via Extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation in rat cardiomyocytes (Germack *et al.*, 2004). The ERK1/2 pathway is also involved in the protective effects of stimulation of A_1 and A_{2B} receptors (Solenkova *et al.*, 2006) and A_{2A} receptors (Kis *et al.*, 2003) in the rabbit heart. It is possible that in presence of endogenous adenosine downstream activation of ERK1/2 pathway through A_1, A_{2A} and A_{2B} adenosine receptors may play an important role in ischemic protection. However, given the lack of any protective effect of A_2 agonists alone, the theory of shared signalling pathways may not explain the co-operative effects observed by Lasley et al (2007) and in the present study. Direct receptor interactions may also explain the A_1/A_2 receptor synergy observed; we are currently investigating these hypotheses, which is described in Chapter 4 in this thesis) (Urmaliya *et al.,* 2010b).

CHAPTER 4

Cooperative cardioprotection via adenosine A_1 and A_{2A} receptor agonism in ischemia-reperfused isolated mouse heart

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4.1. Abstract

In the present study, we investigated a cooperative synergistic cardioprotective effect between adenosine A_1 and A_2 receptors in ischemia-reperfused isolated mouse hearts when CPA was given briefly at reperfusion. Adenosine A_{2A} receptor knockout (A_{2A}KO) and wild-type (WT) mouse hearts (n=9-11) were subjected to global ischemia (30 min) and reperfusion (60 min) in presence of CPA or A_{2A} receptor agonist CGS21680 for the initial 15 min of reperfusion. In WT hearts, CPA (100 nM) significantly ($P<0.05$) improved contractility (52.69 ± 6.22 vs. 23.94 ± 4.93% of baseline), left ventricular developed pressure, end diastolic pressure (EDP), reduced infarct size (7.86 ± 1.73 vs. 23.94 ± 6.62% area at risk), decreased lactate dehydrogenase efflux and increased ERK1/2 phosphorylation at 60 min of reperfusion. Adenosine A_{2A} (ZM241385) and A_{2B} (MRS1754) receptor antagonists abolished CPA-mediated cardioprotection in WT groups, similar to the A_1 receptor antagonist DPCPX. In A_{2A}KO hearts, CPA did not improve functional recovery and protective signaling, with the exception of EDP. Adenosine A_1 receptor-mediated cardioprotection was abolished by adenosine A_{2A}/A_{2B} receptor antagonists or in mice lacking A_{2A} receptors. In this clinically relevant model of pharmacological intervention, pERK-dependent A_1-mediated cardioprotection requires a cooperative activation of A_2 receptors, presumably via endogenous adenosine.

4.2. Introduction

Increased extracellular adenosine during hypoxic conditions (Van Wylen *et al.,* 1992; Headrick 1996) acts on A_1, A_{2A}, A_{2B} and A_3 adenosine receptors (Fredholm *et al.*, 2001), and plays an important role in protection against myocardial ischemia-reperfusion damage (Babbitt *et al.*, 1989; Lasley *et al.*, 1992). Attempts to utilize this protective mechanism in the clinic, using adenosine itself (Mahaffey *et al.*, 1999) or an adenosine A_1/A_2 agonist (Kopecky *et al.*, 2003), have resulted in modest protective effects which were deemed insufficient for further clinical development. For optimal cardioprotection, a greater understanding of the mechanisms involved in adenosine receptor-mediated cardioprotection is required.

Activation of the A_1 adenosine receptor plays a major role in the phenomenon of preconditioning, in which a prior exposure to ischemia reduces the damage that occurs with a later, profound ischemia (Headrick 1996). A_1 receptor occupancy during the myocardial ischemic period improves postischemic functional recovery and protects against vascular dysfunction in isolated rat heart (Lasley *et al.*, 1992) and mouse heart (Flood *et al.*, 2002).

Reperfusion injury has long been recognized as a clinical condition (Buckberg 1987; Piper *et al.,* 1998; Piper *et al.,* 2004) which causes additional injury to the myocardial area at risk (Vinten-Johansen *et al.*, 2007). Intervention at reperfusion is highly desirable in the clinical setting as therapy can be given with thrombolysis in a controlled manner. Given the efficacy of A_1 agonism during ischemia, much attention has focused on A_1 agonist intervention initiated at the time of reperfusion; unfortunately the outcomes have varied

greatly from one study to the next, even in the same species and using similar paradigms. Adenosine administered at the start of reperfusion has been shown to improve functional recovery by activation of A_1 receptors (Donato *et al.*, 2003). Despite some evidence to the contrary (Vander Heide *et al.*, 1996), a number of studies show that activation of A_1 receptors by exogenous agonists during reperfusion ("pharmacological postconditioning") minimizes ischemic contracture and cardiac dysfunction in isolated mouse and rat hearts (Finegan *et al.,* 1996; Lozza *et al.,* 1997; Matherne *et al.,* 1997; Butcher *et al.,* 2007). In contrast, exogenous agonist-mediated A_1 activation had no cardioprotective effect in the hands of other investigators when infused at reperfusion in *in-vivo* rabbit hearts (Thornton *et al.*, 1992; Zhao *et al.*, 1994). There is no obvious reason for the conflicting data, other than the range of agonists and antagonists used, the concentrations of each used, the range of species used, and the time of onset of agonist treatment.

Synergy between A_1 and A_2-mediated cardioprotection has emerged as an important component of adenosine-mediated cardioprotection. The first evidence, from Lasley and colleagues, found that in a preconditioning paradigm, the A_1 -mediated cardioprotective effect was blocked by the adenosine $A_{2A/2B}$ receptor antagonism in *in-vivo* rat heart (Lasley *et al.*, 2007). In support of this, we reported that activation of A_{2A} and/or A_{2B} receptors by endogenous adenosine was required for full A_1-mediated protection to occur in a cardiac cell ischemia model (Urmaliya *et al.*, 2009). Interactions between A_1 and A_2 receptors are well known in the field of neuropharmacology; the inhibitory effect of A_1 agonist N^6-cyclopentyladenosine (CPA) on spike amplitude potential has been shown to be blocked by the A_{2A} agonist 2-*p*-(2-carboxyethy)phenethy-lamino-5'-*N*-ethylcarboxamidoadenosine

(CGS21680) in *in-vitro* rat hippocampus, and this apparent antagonism was reversed by the A_{2A} antagonist ZM241385 (O'Kane *et al.*, 1998). The A_1 agonist (CCPA)-mediated antiadrenergic effect has been shown to be inhibited by A_{2A} antagonist ZM241385 (Tikh *et al.*, 2006). No report to date shows whether A_1/A_2 synergy occurs in the most clinically relevant setting of pharmacological cardioprotection; intervention at reperfusion, hence this is the focus of the present study.

Pro-survival kinase activation has been shown to produce cardioprotection against reperfusion injury in various animal models (Hausenloy *et al.*, 2004). Adenosine A_1, A_{2A}, A_{2B} and A_3 receptors activate pro-survival kinase through increased extracellular signal-regulated kinase (ERK) 1/2 phosphorylation in CHO cells (Schulte *et al.*, 2000), rat cardiomyocytes (Germack *et al.*, 2004), isolated mouse hearts (Morrison *et al.*, 2007) and *in-vivo* rat hearts (Reid *et al.*, 2005). As both A_1 and A_2 receptors exert cardioprotective effects via ERK1/2 activation, it is possible that there is some synergy of function at this level.

In the present study we investigated the role of adenosine A_2 receptor activation in adenosine A_1-mediated cardioprotection against ischemia-reperfusion injury using both pharmacological and targeted gene knockout interventions in isolated mouse hearts. Given that A_1 and A_{2A} synergistic effects are unlikely to be exerted at the level of adenylate cyclase and cAMP, on which these two receptors have opposing effects, we hypothesized that the synergistic benefits in a clinically relevant model of A_1 agonist intervention occurs

when CPA is only added briefly at reperfusion, may relate to a more than additive effect of A_1 and A_2 activation on ERK1/2 phosphorylation.

4.3. Materials and Methods

4.3.1. Animals

All animal experiments were conducted follows the requirements from the Prevention of Cruelty to Animals Regulations, 2008, under the guidelines of the Australian National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Scientific Purposes in Australia. The adenosine A_{2A} receptor knockout (A_{2A}KO) and wild type (WT) male adult mice used in the present study were bred on a CD-1 background, originally provided and characterized by Ledent and colleagues (Ledent *et al.*, 1997). Mice were routinely genotyped by PCR (Snell *et al.*, 2000), as described in the supplementary data (Supplementary Figure 4.1). WT and A_{2A}KO colonies were bred and maintained in-house at the Monash Institute of Pharmaceutical Sciences animal house at 22 ºC with a constant 12 hour light/12 hour dark cycle. Animals had free access to mouse chow and water *ad libitum.* The experimental protocols were formally approved by the Monash University Standing Committee of Animal Ethics in Animal Experimentation, Faculty of Pharmacy and Pharmaceutical Sciences, Monash University, Parkville campus, Australia (# RPW 2008/02).

4.3.2. Langendorff-perfused isolated mouse heart model

Langendorff-perfused isolated heart preparation in mice was carried out as previously reported (Headrick *et al.*, 2001a), and described in the supplementary data. Male mice (8-16 weeks of age) were anesthetized with sodium pentobarbital (60mg/kg, intraperitoneal injection), a thoractomy was performed and the hearts were rapidly excised into ice cold perfusion buffer. The hearts were retrogradely perfused through the aorta at a constant

pressure of 80 mmHg with modified Krebs-Henseleit buffer. A fluid-filled balloon was used to measure left ventricular systolic and diastolic pressure. The heart was submerged in to the Krebs-Henseleit buffer maintained at 37^0C throughout the experiment. Continuous in-line coronary perfusion flow and temperature were measured and recorded, along with the coronary perfusion pressure, by PowerLab data acquisition system (ADInstruments, Bella Vista, NSW, Australia). The left ventricular pressure signals acquired continuously using a PowerLab and digitally processed using ChartPro vs5.5.6 software (AD Instruments) were used to measure left ventricular systolic and diastolic pressure and heart rate. Differences between systolic and diastolic pressures were used to obtain left ventricular developed pressure (LVDP) and its first derivative, dP/dt_{max}.

4.3.3. Global ischemia-reperfusion technique

After 30 min stabilization, baseline functional measurements were acquired immediately before hearts were subjected to 30 min of no-flow normothermic global ischemia and 60 min of reperfusion (I-R). Ischemia was attained by completely stopping the perfusate inflow and immersing the heart in perfusate buffer which was saturated with 95% N_2 + 5% O_2 in the organ bath. After the ischemic period, reperfusion was achieved by opening the perfusion in-line flow.

4.3.4. Experimental groups and design

Figure 4.1 shows the experimental groups used for both WT and A_{2A}KO isolated hearts. To examine the involvement of adenosine A_1, A_{2A} and A_{2B} receptors on functional recovery, hearts were perfused with buffers containing the indicated agonist and antagonist

concentrations for the initial 15 min of reperfusion. When antagonists were used, they were perfused for 5 min before reperfusion was initiated, at which point both agonist and antagonist were added for the first 15 min of reperfusion. In the present study the I-R protocol was selected based on empirical studies performed in our laboratory (data not shown) for different time points; the conditions chosen were such that significant but not complete necrosis occurred. 9-11 WT and A_{2A}KO mice per treatment group were used in this study, and functional data was obtained for all hearts. A subset of hearts for each treatment group was used to determine infarct size (5-6 per group) and a separate subset (5 per group) used to determine ERK1/2 levels.

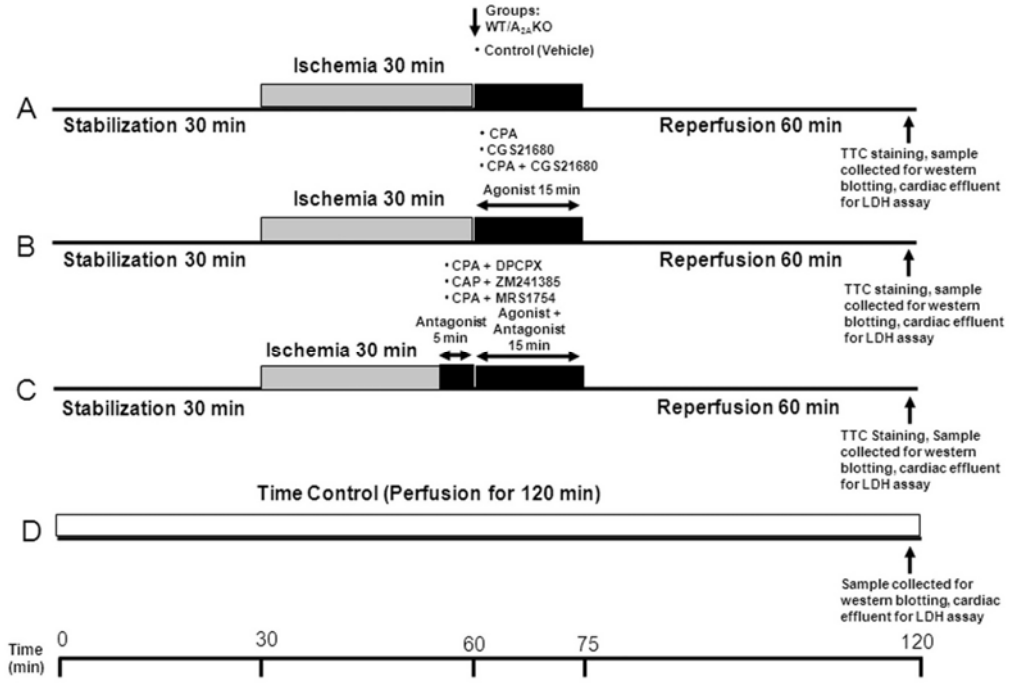


Figure 4.1. Experimental protocol for ischemia and reperfusion damage in WT and A_{2A}KO mice groups in Langendorff perfused isolated mouse heart model. Group 1 time control isolated hearts were perfused for 120 min without I-R; Group 2 Control, isolated hearts were exposed to I-R; Group 3 CPA (100nM), isolated hearts were exposed to I-R with

CPA; Group 4 CGS21680 (100nM), isolated hearts were exposed to I-R with CGS21680; Group 5 CPA (100nM) + CGS21680 (100nM), isolated hearts were exposed to I-R with CPA + CGS21680; Group 6 CPA (100nM) + DPCPX (1 μM) isolated hearts were exposed to I-R with DPCPX + CPA; Group 7 CPA (100nM) + ZM241385 (50nM), isolated hearts were exposed to I-R with ZM241385 + CPA; Group 8 CPA (100nM) + MRS1754 (100nM) isolated hearts were exposed to I-R with MRS1754 + CPA.

4.3.5. Infarct size measurement

At the end of the experiment, a subset of hearts were quickly removed, weighed, wrapped in a plastic film and placed at -20^{0}C for 5-6 hrs. The frozen hearts were sectioned into 7-8 transverse slices of approximately equal thickness of 1.5 mm. They were stained by incubation in 1% 2, 3, 5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich, St Louis, MO, USA) for 15 min at 37^{0}C. After fixing in 10% formalin saline for overnight, heart sections were scanned using a flatbed scanner (K10291, Canon Scan LiDE 600F). The area of infarction and risk zone was measured using ImageJ 1.41o software (National Institute of Health, USA). The risk area was calculated as total ventricular area minus cavities. Infarct size is expressed as a percentage of the risk area.

4.3.6. Lactate dehydrogenase (LDH) release from cardiac effluent

Ischemia-reperfusion injury-induced cellular damage was evaluated in WT and A_{2A}KO mice hearts by the measurement of LDH release from cardiac effluent collected at the end of reperfusion. LDH release was measured by using a commercial kit, CytoTox® 96 Non-Radioactive Cytotoxicity Assay (Promega, Madosin, WI, USA) as per manufacturer

guidelines. CytoTox® 96 Non-Radioactive Cytotoxicity Assay is a colorimetric assay quantitatively measures LDH released upon cell lysis or damage. LDH released in a mouse cardiac effluent during ischemia reperfusion injury was measured with a 30 min coupled enzyme assay, which result in the conversion of a tetrazolium salt into a red formazan product. The amount of color formed is proportional to the percentage damage of cardiac cells, which was expressed as percentage cytotoxicity.

4.3.7. ERK 1/2 phosphorylation by western blot analysis

At the end of reperfusion a subset of hearts were homogenized and lysed in protein lysis buffer and processed to obtain protein samples (Hollande *et al.*, 2001), as described in supplementary data. 50µg of total protein was diluted with 1:1 loading buffer and denatured and separated using 10% SDS-PAGE and transferred on to a nitrocellulose membrane using the Trans-Blot semi-dry blotting system (Bio-RAD, CA, USA). The membrane was then incubated with primary antibody for total ERK1/2 (catalogue no. CS9102, Cell Signaling Technology, Danvers, MA, USA) and phosphorylated ERK (pERK) 1/2 (catalogue no. CS4370, Cell Signaling Technology) at 1:1000 dilution. Fluorescently-labeled secondary antibodies (926-32212 IRDye 800CW Donkey anti-mouse IgG and 926-3223 IRDye 680CW Donkey anti-rabbit IgG, LI-COR Bioscience, Nebraska, USA) were used at 1:20000 dilution to visualize immunoblots via an Odyssey infrared imaging system (LI-COR Bioscience). Densitometric analysis of protein bands was performed using Image J software version 1.41o (National Institute of Health, USA). Data were expressed as a ratio of phosphorylated kinase to total kinase signal.

4.3.8. Drugs and stock solutions

Stock solutions were prepared in dimethyl sylfoxide (DMSO) for all agonists and antagonists containing no more than 0.1% DMSO and stored at -20^{0}C. At the time of experiment stock solutions were freshly diluted in perfusion buffer to make the required concentration. CPA, DPCPX, MRS1754 were purchased from Sigma-Aldrich (Castle Hills, NSW, Australia). CGS21680 and ZM241385 were purchased from Tocris Biosciences (Ellisville, Missouri, USA).

4.3.9. Statistical analysis

The data were presented as a mean ± SEM. Cardiac functional data at baseline and after ischemia-reperfusion in WT and A_{2A}KO mice groups were analyzed by two-way ANOVA followed by Bonferroni's post hoc test. Cardiac functional data at 60 min of reperfusion, LDH release, infarct size measurement and ERK1/2 western blot data from WT and A_{2A}KO hearts were analyzed by one-way ANOVA followed by Bonferroni's post hoc test. $P < 0.05$ was considered statistically significant.

4.4. Results

4.4.1. Baseline functional data

Average wet heart to body weight ratio%, heart rate and coronary flow at the end of stabilization were not different between WT and A_{2A}KO hearts (Table 4.1).

Table 4.1. Baseline functional data in WT and A_{2A}KO mice subjected to global ischemia (30 min) followed by reperfusion (60 min)

Groups	Wet heart to body weight ratio (%)		Heart rate (beats per min)		Coronary flow (ml/min)	
	WT (n= 9-11)	**A_{2A}KO** (n= 9-11)	**WT** (n= 9-11)	**A_{2A}KO** (n= 9-11)	**WT** (n= 9-11)	**A_{2A}KO** (n= 9-11)
Control	0.74 ± 0.03	0.74 ± 0.01	353.57 ± 26.78	329.03 ± 13.98	3.45 ± 0.34	2.66 ± 0.57
CPA (100 nM)	0.62 ± 0.02	0.62 ± 0.04	376.37 ± 9.42	355.01 ± 4.56	3.12 ± 0.37	3.18 ± 1.23
CGS21680 (100 nM)	0.69 ± 0.03	0.68 ± 0.03	451.33 ± 51.81	525.54 ± 60.22	2.14 ± 0.12	2.33 ± 0.58
CPA (100 nM) + CGS21680 (100 nM)	0.69 ± 0.04	0.69 ± 0.02	361.25 ± 12.98	430.28 ± 67.76	2.43 ± 0.17	1.69± 0.29
CPA (100 nM) + DPCPX (1 μM)	0.72 ± 0.05	0.60 ± 0.03	418.22 ± 72.65	288.21 ± 30.41	3.02 ± 0.29	2.67± 0.56
CPA (100 nM) + ZM241385 (50 nM)	0.68 ± 0.03	0.62 ± 0.03	357.30 ± 19.09	395.68 ± 14.32	2.52 ± 0.27	3.41 ± 0.62
CPA (100 nM) + MRS1754 (100 nM)	0.67 ± 0.03	0.62 ± 0.04	420.15 ± 44.44	395.60 ± 50.23	2.55 ± 0.23	1.98 ± 0.23

4.4.2. Cardiac contractility (dP/dt_{max}) and left ventricular developed pressure (LVDP) measurement

Figures 4.2 and 4.3 (A,B) show changes in contractility and LVDP during the ischemia-reperfusion protocol for control and CPA-treated hearts. In WT hearts treated with CPA (100 nM), functional recovery was enhanced compared to vehicle control (I-R) hearts as assessed by dP/dt_{max} and LVDP at 60 min of reperfusion (52.69 ± 6.22 vs. 23.94 ± 4.93 % of baseline and 54.73 ± 7.27 vs. 18.74 ± 4.45 % of baseline respectively, n= 9-11, $P<0.05$, Figure 4.2C, 4.3C). In WT hearts, CPA-mediated improvement in both LVDP and dP/dt_{max} were significantly blocked in the presence of DPCPX (1μM), ZM241385 (50 nM) or MRS1754 (100 nM) (LVDP data: 54.73 ± 7.27 vs. 19.14 ± 5.33, 32.57 ± 7.80, 32.69 ± 4.36 % of baseline respectively, $P<0.05$, Figure 4.3C). In WT hearts, CGS21680 (100 nM) and CPA (100 nM) together did not improve dP/dt_{max} compared to control (Figure 4.2C; $P>0.05$), unlike CPA alone. In A_{2A}KO hearts CPA had no effect on either LVDP or dP/dt_{max} recovery (n= 9-11, $P>0.05$; Figure 4.2B, D, Figure 4.3B, D).

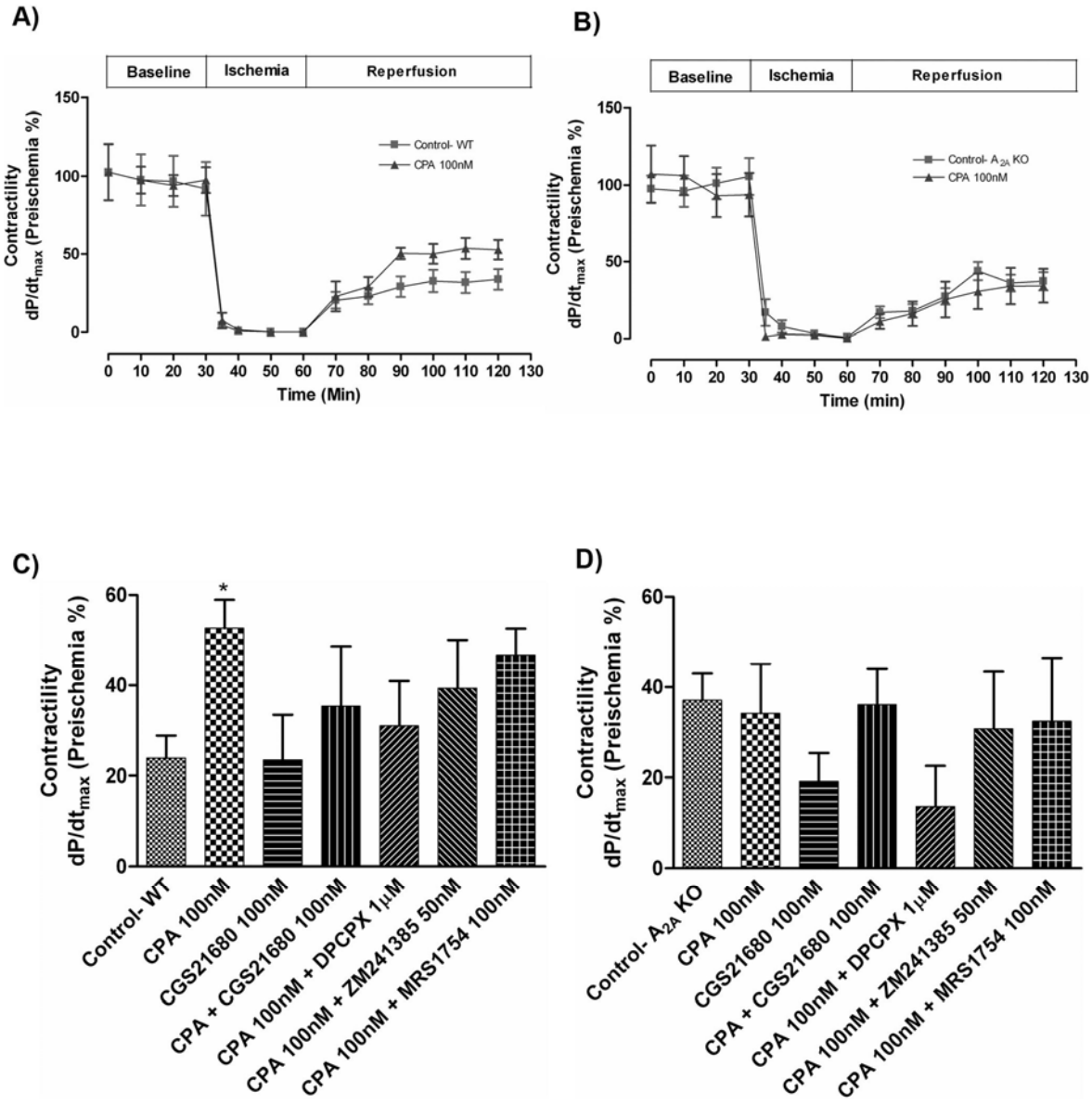


Figure 4.2. Contractility (dP/dt_{max}) in WT (A) and A_{2A}KO (B) mouse hearts during global ischemia and reperfusion, in the presence and absence of CPA 100 nM. C and D show post ischemic dP/dt_{max} in WT (C) and A_{2A}KO (D) mice hearts at a single time point at the end of reperfusion in the presence of CPA, CGS21680, CPA + CGS21680 alone or in combination with DPCPX or ZM241385 or MRS1754. The data were normalized to baseline and

expressed as pre-ischemic percentage (mean ± SEM, n= 9-11, P < 0.05, *significantly different to control (I-R).

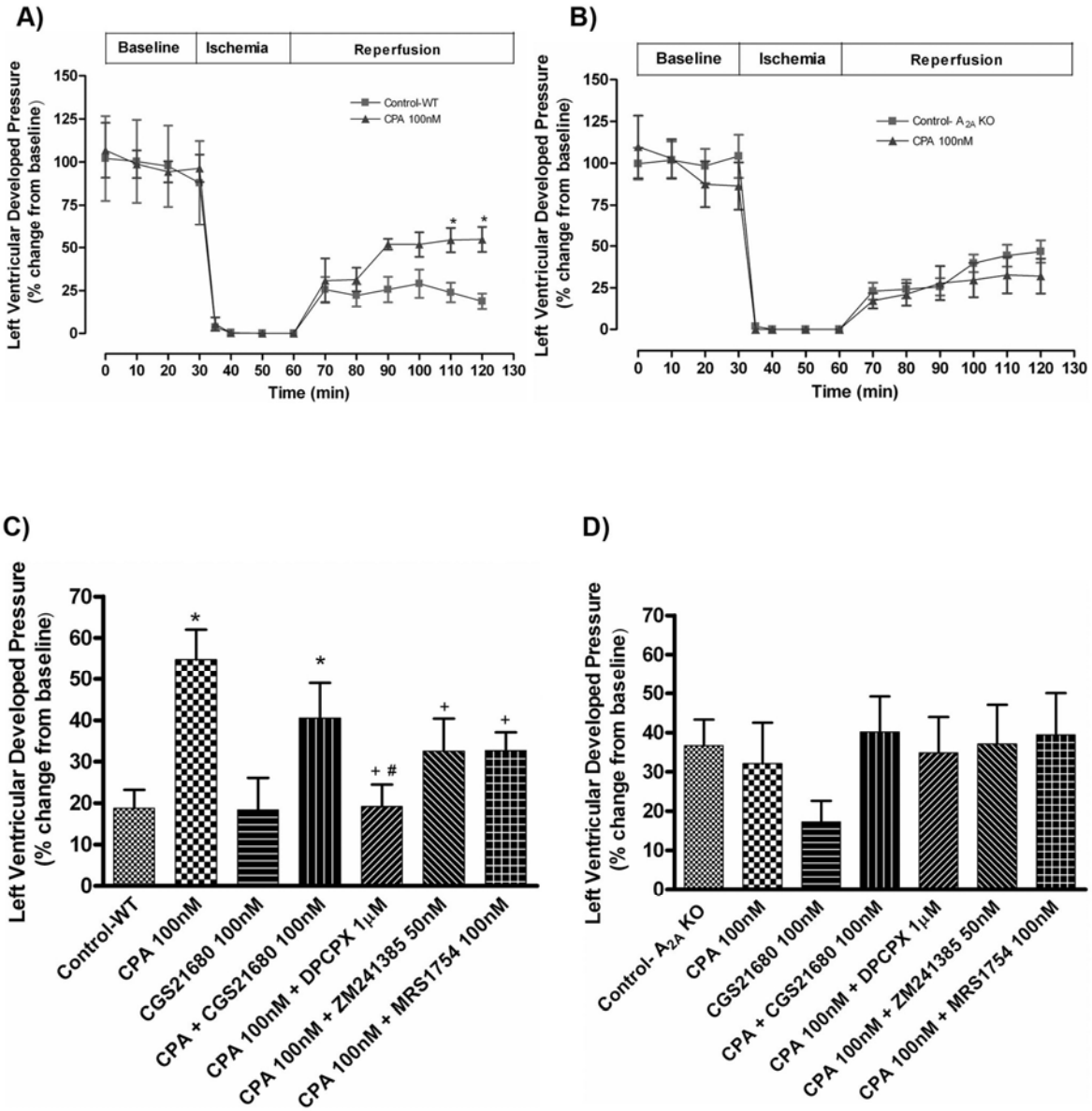


Figure 4.3. Left ventricular developed pressure (LVDP, mmHg) recovery in WT (A) and A_{2A}KO (B) mouse hearts during global ischemia and reperfusion in the presence and absence of CPA 100 nM. C and D show post ischemic LVDP in WT (C) and A_{2A}KO (D) mice hearts at a single time point at the end of reperfusion in the presence of CPA,

CGS21680, CPA + CGS21680 alone or in combination with DPCPX or ZM241385 or MRS1754. The data were normalized to baseline values and expressed as pre ischemic percentage (mean ± SEM, n= 9-11, P < 0.05, *significantly different to control (I-R), $^{+}$significantly different to CPA, $^{\#}$ significantly different to CPA + CGS21680.

4.4.3. End diastolic pressure (EDP) measurement

EDP rose after the onset of ischemia, a phenomenon known as contracture. After 10 min of ischemia, EDP was increased significantly in A_{2A}KO compared to WT hearts (77.99 ± 6.85 vs. 47.98 ± 12.19 mmHg respectively, n=9-11, P<0.05, Figure 4.4A, B, trace shown in supplementary Figure 4.2). At the end of reperfusion CPA (100 nM) alone and/or combination with CGS21680 (100 nM) significantly reduced ischemic contracture compared to control in WT hearts (25.79 ± 6.06, 17.54 ± 6.94 vs. 49.68 ± 1.14 mmHg respectively, P<0.05), an effect blocked in the presence of DPCPX (1 μM), MRS1754 (100 nM) and ZM241385 (50 nM) (25.79 ± 6.06 vs. 45.64 ± 2.03 mmHg for CPA alone and 17.54 ± 6.94 vs. 45.64 ± 2.03, 39.21 ± 3.63, 35.49 ± 6.13 mmHg for CPA + CGS21680 respectively, P<0.05, Figure 4.4C). In A_{2A}KO hearts contracture was significantly reduced with CPA at 30, 50 and 60 min of reperfusion (40.76 ± 4.61, 36.07 ± 5.06, 35.96 ± 5.44 vs. 10.64 ± 2.40, 6.67 ± 2.31, 6.41 ± 2.28 mmHg respectively, P <0.05, Figure 4.4B). DPCPX and MRS1754, unlike ZM241385 significantly blocked the CPA-mediated effect in A_{2A}KO hearts (30.51 ± 7.32, 25.01 ± 5.03 vs. 6.41 ± 2.28 mmHg respectively, P <0.05, Figure 4.4D).

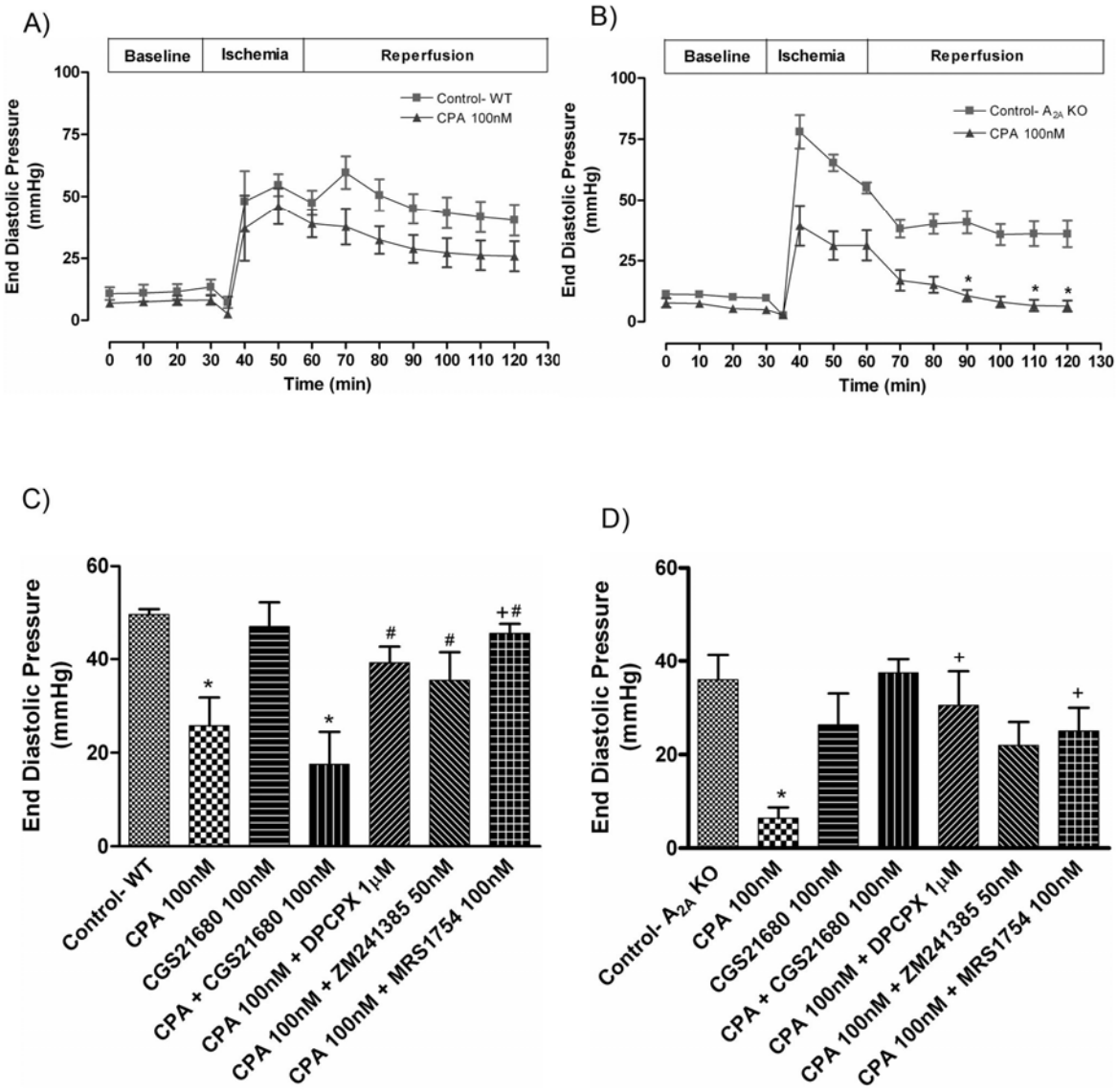


Figure 4.4. End diastolic pressure (EDP, mmHg) in WT (A) and A_{2A}KO (B) mouse hearts during global ischemia and reperfusion in the presence and absence of CPA 100 nM. Post ischemic EDP in WT (C) and A_{2A}KO (D) mouse hearts at a single time point at the end of reperfusion in the presence of CPA, CGS21680, CPA + CGS21680 alone or in combination with DPCPX or ZM241385 or MRS1754. The data are expressed as mean ± SEM (n= 9-11, $P < 0.05$, *significantly different to control (I-R), $^{+}$significantly different to CPA, $^{\#}$significantly different to CPA + CGS.

4.4.4. Infarct size and lactate dehydrogenase (LDH)

Infarct size was significantly reduced in WT mouse hearts in the presence of CPA (100 nM) compared to ischemic control hearts (7.86 ± 1.73% area at risk for CPA vs. 23.94 ± 6.62% area at risk for control respectively); an effect reversed by DPCPX (1μM) and ZM241385 (50 nM) or MRS1754 (100 nM) (32.63 ± 2.79% area at risk for CPA + DPCPX; 41.56 ± 3.39% area at risk for CPA + ZM241385; 34.61 ± 2.77% area at risk for CPA + MRS1754, n=6, $P<0.05$, Figure 4.5A). LDH release significantly increased in control (I-R) hearts compared to time control (no ischemia) hearts in both WT and A_{2A}KO hearts (16.95 ± 1.77 vs. 2.57 ± 0.49% cytotoxicity and 18.04 ± 0.93 vs. 2.66 ± 0.34% cytotoxicity, n=5, $P<0.05$, Figure 4.5C, D). CPA alone or combination with CGS21680 (100 nM) significantly reduced LDH release compared to the control hearts from WT mice (8.28 ± 0.74, 10.40 ± 0.55 vs. 16.95 ± 1.77% cytotoxicity respectively, $P<0.05$), an effect reversed in the presence of DPCPX or ZM241385 or MRS1754 (8.28 ± 0.74, 10.40 ± 0.55 vs. 18.45 ± 1.09, 18.57 ±1.61, 16.80 ± 1.20% cytotoxicity respectively). CPA alone or in combination with CGS21680 did not show any significant effect ($P>0.05$) on infarct size or LDH release in A_{2A}KO hearts. However, MRS1754 (100 nM) in the presence of CPA compared to CPA alone significantly increased infarct size in A_{2A}KO mice (41.77 ± 2.33% area at risk for CPA + MRS1754, $P<0.05$, Figure 4.5B).

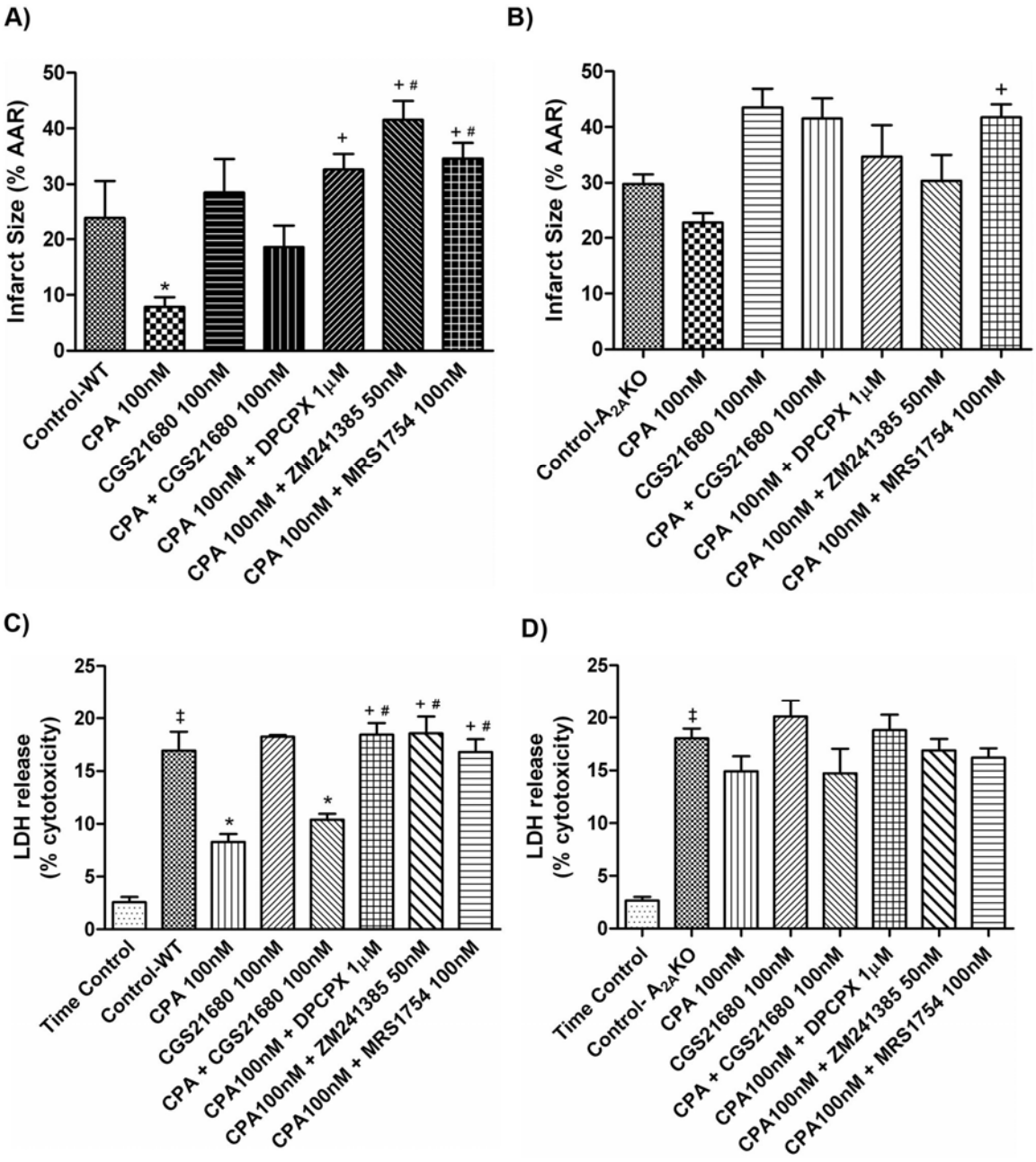


Figure 4.5. Myocardial infarct size measurement and lactate dehydrogenase (LDH) release from cardiac effluent in WT (A, C) and A_{2A}KO (B, D) mouse hearts after global ischemia and reperfusion in the presence of CPA, CGS21680 or CPA + CGS21680 alone or in combination with DPCPX or ZM241385 or MRS1754. The infarct size data (A, B) is expressed as % area at risk. LDH samples were collected at 60 min of reperfusion, and data

represented as percent cytotoxicity (C, D; mean ± SEM, n=5-6, P<0.05, ‡significantly different to time control, *significantly different to control (I-R), +significantly different to CPA, #significantly different to CPA + CGS.

4.4.5. Post ischemic ERK1/2 phosphorylation

In WT hearts a significant increase in ERK1/2 phosphorylation was observed with CPA (100 nM) treatment (1.53 ± 0.26 for CPA vs. 0.83 ± 0.06 for control I-R, n=5, P<0.05), an effect which was significantly blocked in the presence of DPCPX (1μM) and ZM241385 (50 nM) (0.092 ± 0.16 for CPA + DPCPX, and 0.83 ± 0.05 for CPA + ZM241385, P<0.05, Figure 4.6A). In A_{2A}KO hearts ERK1/2 phosphorylation was not affected by either CPA alone or in combination with CGS21680 compared with A_{2A}KO control (I-R) hearts (0.95 ± 0.24 for CPA, 0.84 ± 0.06 for CGS, 1.09 ± 0.05 for CPA + CGS vs. 0.75 ± 0.15 for control (I-R), P>0.05, Figure 4.6B).

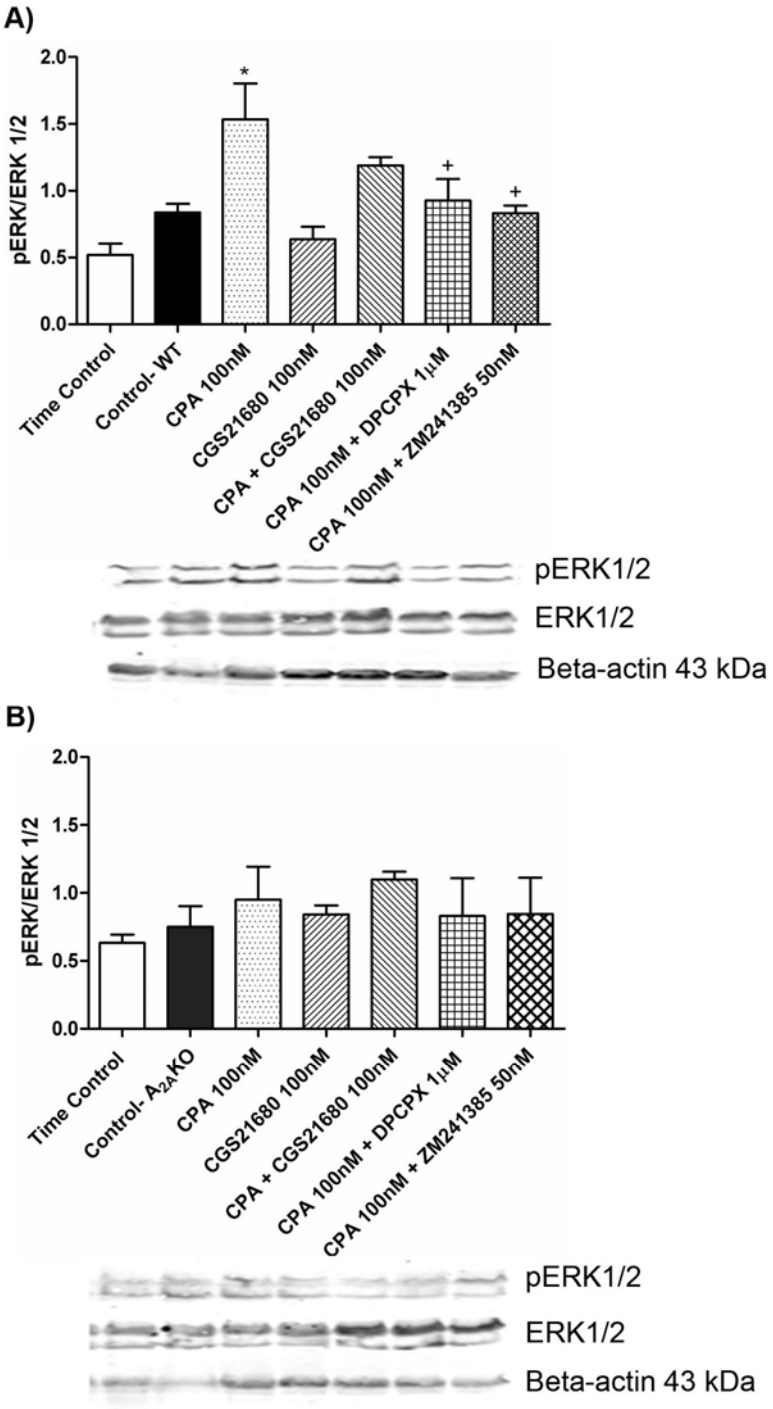


Figure 4.6. Extracellular signal-regulated kinase (ERK) 1/2 phosphorylation in WT (A) and A_{2A}KO (B) mouse isolated heart tissue homogenates after global ischemia and reperfusion. Hearts were exposed to ischemia-reperfusion injury in presence of CPA,

CGS21680 or, CPA + CGS alone or in combination with DPCPX or ZM241385. (A) and (B) bottom shows representative western blots for phosphorylated ERK1/2, total ERK1/2 and beta-actin control. ERK1 and ERK2 bands were combined to obtain total ERK density. The data is expressed as the ratio of phosphorylated ERK 1/2 to total ERK1/2 (mean ± SEM, n= 5, $P<0.05$, *significantly different to control (I-R), $^{+}$significantly different to CPA.

4.5. Discussion

Previous reports of synergistic cardioprotection from adenosine A_1 and A_2 receptor activation occurred in paradigms in which the agonist was introduced during ischemia. In order for this phenomenon to have clinical relevance, A_1 / A_2 synergy would need to be observed in models in which the agonist was added at reperfusion. Importantly, the data from the present study show that A_1 agonist-mediated protection at reperfusion in mouse hearts is completely abolished when adenosine A_2 receptor activation is prevented. These data rule out the possibility that the previous conclusions of A_1 / A_2 cardioprotective interactions were a result of a lack of specificity of the pharmacological tools employed, as there was excellent agreement between knockout and pharmacological intervention studies.

In the present study, we demonstrated that CPA infusion for 15 minutes at the onset of reperfusion is cardioprotective in terms of improved postischemic cardiac functional recovery (increased contractility- dP/dt_{max} and left ventricular developed pressure- LVDP), cell death, and reduced ischemic contracture (end diastolic pressure- EDP) in WT isolated hearts. However, in A_{2A}KO mice, CPA infusion at the onset of reperfusion failed to give cardioprotection. The literature in the field is divided over the effectiveness of A_1 protection when given at reperfusion. Whilst many studies show that A_1 activation during ischemia produces cardioprotection (Lasley *et al.,* 1990; Lasley *et al.,* 1992; Zhao *et al.,* 1994), there are conflicting reports as to whether A_1 occupancy during reperfusion is protective.

Supporting evidence for A_1 protection at reperfusion comes from studies in isolated mouse (Matherne *et al.*, 1997; Peart *et al.*, 2000), rat (Finegan *et al.*, 1996; Lozza *et al.*, 1997) and rabbit hearts (Donato *et al.,* 2007b). There are, however, a number of studies showing no protective effect of A_1-agonists when given at reperfusion. Variously, it has been suggested that cardioprotection is mediated by activation of A_{2A} and A_3 receptors but not A_1 receptors in *in-vivo* rat heart (Kin *et al.,* 2005b) and A_3 but not A_1 and A_{2A} receptors in isolated mouse heart (Peart *et al.*, 2002) ischemia-reperfusion models. In an unusual, multi-centre study in rabbits, the overall outcome of numerous experiments was that A_1 agonism at reperfusion was not protective (Baxter *et al.*, 2000), although of note there was significant variability in outcomes at the different centers in which the study was conducted. There are many differences in animal model, drug concentration used and timing of agonist addition between the reports of successful and unsuccessful A_1 protection at reperfusion, making it difficult to compare one study to another. What is clear is that well-controlled studies have shown both protection and a lack of protection from exogenous A_1 agonists at reperfusion. In an attempt to avoid being distracted by species differences and to therefore focus on the clinical relevance of A_1 activation at reperfusion, we are currently investigating the nature of A_1-mediated protective effects in human cardiomyocytes.

An exception to the requirement for A_2 receptor activation for full A_1 protection was found in the case of contracture: the rise in end diastolic pressure that occurs during ischemia. A_1-mediated reduction in contracture is dependent upon mitochondrial K_{ATP} channels (Headrick *et al.,* 2000). CPA produced a significant reduction in EDP both during ischemia (i.e. prior to CPA treatment) and at the end of reperfusion in CPA-treated A_{2A}KO mice

compared to A_{2A} KO control mice, as well as in CPA treated WT mice compared to control. The reduced EDP during ischemia in this group is a finding difficult to explain; perhaps a consequence of several outlier data points in the control group. Nonetheless, the results of this study show no evidence of the requirement for A_2 receptor activation in A_1-mediated reductions in contracture.

In the present study we demonstrated that A_1 mediated postischemic cardiac functional recovery, infarct size reduction and LDH release in coronary effluent was abolished not only by the adenosine A_1 antagonist DPCPX, but also by A_{2A} and A_{2B} selective antagonists (ZM241385 and MRS1754 respectively) in WT isolated heart. In A_{2A}KO mice the addition of CPA was not protective by any of these measures. We observed reduced EDP during ischemia (i.e. prior to CPA treatment) in CPA-treated A_{2A}KO mice compared to A_{2A} KO control mice, a finding difficult to explain; perhaps a consequence of several outlier data points in the control group. These data suggest that synergistic protection occurs at reperfusion when A_1 receptors are activated by CPA at the same time that A_{2A}/A_{2B} receptor subtypes are activated, presumably by endogenous adenosine. Notably, A_{2A} receptor activation alone was insufficient to cause measurable protection, in agreement with previous studies using isolated rat heart (Finegan *et al.*, 1996; Maddock *et al.*, 2002) or *in-vivo* rat heart during ischemia (Lasley *et al.*, 2007) or rat isolated ventricular cardiomyocyte (Kilpatrick *et al.*, 2002). Adenosine receptor interactions in ischemia-reperfused mouse (Tikh *et al.*, 2006) and rat hearts (Lopes *et al.,* 1999; Germack *et al.,* 2005; Lasley *et al.,* 2007) have been reported in various studies. Lasley and colleagues recently found that activation of A_{2A} and/or A_{2B} receptors is required for A_1-mediated cardioprotective effect in

in-vivo rat hearts (Lasley *et al.*, 2007), in addition to our work in a cardiac cell ischemic model (Urmaliya *et al.*, 2009). The significance of the present study lies in the fact that activation of both adenosine A_1 and A_2 receptors appears to be required for maximal cardioprotection in a clinically relevant model involving intervention at reperfusion. Future clinical trials in this area should aim to ensure that activation of both receptor subtypes occurs.

The result of the present study should be interpreted with a caveat that the adenosine A_1 and A_{2A} antagonist DPCPX and ZM241385 respectively, have a relatively high binding affinity for the adenosine A_{2B} receptor as well in various rodent species, which has been reported recently (Auchampach *et al.,* 2009). The adenosine A_{2B} antagonist MRS1754 (200 nM) used in the present study has shown some binding affinity for the adenosine A_1 receptor in rat (Table 1.2 B). In hindsight, a lower concentration of DPCPX and MRS1754 would have been appropriate.

A large body of evidence suggests that the downstream activation of ERK1/2 signaling by adenosine receptors is key to cardioprotection arising from activation of adenosine receptors, including reports from studies using isolated rat cardiomyocytes (Germack *et al.*, 2004), in CHO cells expressing human adenosine receptors (Schulte *et al.*, 2000), in isolated mouse heart (Morrison *et al.*, 2007) and *in-vivo* using rat hearts (Reid *et al.*, 2005). We observed that CPA treatment improved cardioprotective signaling concomitant with a significant increase in ERK 1/2 phosphorylation in WT isolated heart. However, CPA treatment did not show any effect on ERK1/2 phosphorylation in A_{2A}KO hearts. CPA-

mediated increases in ERK1/2 phosphorylation signaling were significantly reversed by the A_{2A} antagonist ZM241385 in WT mice. CPA-mediated ERK1/2 phosphorylation signaling was not blocked by treatment with an adenosine A_{2B} selective antagonist in WT isolated hearts. The data suggest that endogenous activation of A_2 receptors is required for full ERK1/2 signaling through A_1 receptors. Interestingly, there was no effect on ERK phosphorylation of the A_{2A} agonist CGS21680 alone, and no extra ERK1/2 phosphorylation in CPA-treated hearts when CGS21680 was added. These data suggest that the A_{2A} synergistic effect revealed using A_{2A} knockout mice or ZM241385 is maximally exerted by the endogenous adenosine present using this model, and cannot be boosted via exogenous agonists.

When considering the implications of our data, it is useful to consider the reports of the adenosine A_1/A_{2A} receptor agonist AMP579. AMP579 limits myocardial infarction in *in-vivo* rabbit heart (Kis *et al.*, 2003) and reduces ischemic contracture and infarct size in isolated rabbit heart when administered during reperfusion (Xu *et al.*, 2001; Liu *et al.*, 2010). Recently, Downey and colleagues reinterpreted the cardioprotective effects of AMP579 to be due to A_{2B} agonism, as they were blocked by A_{2B} selective antagonists MRS1754 and PSB1115 (Liu *et al.*, 2010). Our data raises the possibility that these authors may have actually interfered with A_1/A_2 receptor synergy, and that AMP579 is likely to activate A_1 and A_2 receptors as part of its protective mechanism.

In this study we demonstrate that CPA-mediated adenosine A_1 receptor activation is cardioprotective at reperfusion in WT isolated heart as measured by improved postischemic

functional recovery, decrease in infarct size and cardiac LDH release and increase in ERK1/2 phosphorylation. In contrast, CPA did not have any effect on these indicators of postischemic recovery in A_{2A}KO isolated hearts. Moreover, cardioprotective ERK1/2 signaling was increased in the presence of CPA in WT isolated heart, but not in A_{2A}KO isolated hearts or those receiving ZM241385. These results suggest that adenosine A_1-mediated cardioprotection involves cooperative activation of adenosine A_{2A} and A_{2B} receptors by endogenous adenosine in our isolated mouse heart ischemia-reperfusion model.

4.6. Supplementary materials and methods

4.6.1. Genotyping of knockout mice

4.6.1.2. Composition of standard buffer used

Alkaline Lysis reagent: 25 mM NaOH, 0.2 mM disodium EDTA, pH 12

Neutralizing reagent: 40 mM Tris-HCL, pH 5

GoTaq® Green Master Mix (x1): 200 µM each dNTP, 1.5 mM $MgCl_2$, DNA Taq polymerase, loading dye

Tris acetate ETDA (TAE): 40 mM Tris acetate, 1 mM EDTA

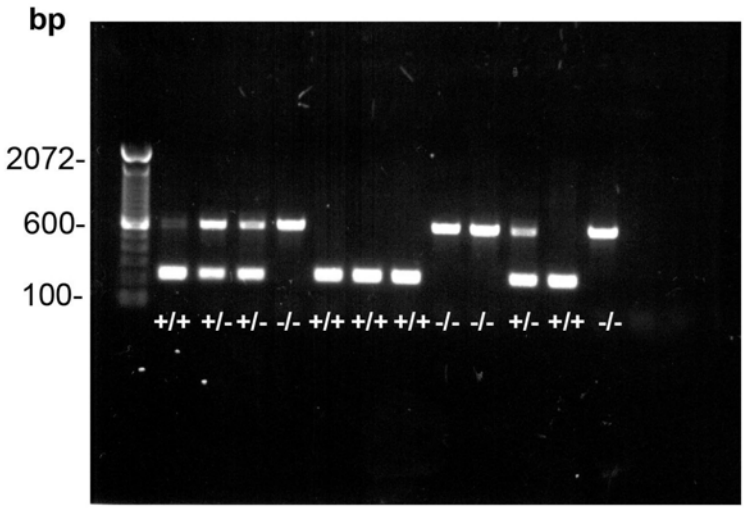
4.6.1.3.Extraction of genomic DNA from tail tissue sample

Mouse genomic DNA was extracted from tail tissue biopsies (1-3 mm tail snips) using Hot Sodium Hydroxide and Tris (HotSHOT) method (Truett *et al.*, 2000). Tissue samples were collected in sterile tubes. Alkaline lysis reagent (600 µl/tube) was added to the tissue samples and heated to 95^{o}C in a heating block for 10 minutes. After heating samples was vortex for 30 seconds at room temperature. After vortex samples were neutralized by adding 150 µl neutralizing reagent to each samples. Sample tubes were centrifuged for 6 min at 12000-13000 rpm to separate the undissolved tissue. One microliter of the final preparation is used per each 25 µl PCR reaction.

4.6.1.4. Polymerase chain reaction (PCR) for mouse genotyping

The mouse genotype was routinely determined by the polymerase chain reaction (PCR). One µl aliquot of HotSHOT DNA was combined with PCR master mix containing 12.5 µl of 2x GoTaq® Green Master Mix (Promega, USA). Forward and reverse primers were

combined at 0.2 µM each with PCR mastermix a specific concentration of each primer, made up to 25 µl with nuclease free water (Promega, USA) to detect mutant and normal strains. Reactions were amplified in a 2720-thermal cycler™ (Applied Biosystem, USA) for 30 cycles at 94^{o}C for 45 sec and 55^{o}C for 45 sec. Following amplification, 15 µl of each sample were loaded on 2% TAE- agarose gel containing SYBR® safeDNA gel stain (10,000X, Invitrogen, USA) diluted it to 1:10,000 in 1X TAE agarose gel buffer, in an electrophoresis gel tank (Bio-rad Mini Sub® Cell GT, USA). The samples were electrophoresed at 60 V for 60 min with 100 base pair DNA ladder (Tracklt™, Invitrogen) and the resultant bands visualized and photographed under ultra violet (UV) light using a GeneFlash Syngene Bio Imaging and Video Graphic Printer UP-895MD system (WI, USA) as shown in supplementary Figure 4.1.



Supplementary Figure 4.1. Mouse genotyping-polymerase chain reaction (PCR) image. A single band at 229 and 570 base pairs (bp) shows wild type and A_{2A} knockout mice respectively. Lane with both bands show heterozygous mice. First lane indicates a 100 bp reference DNA ladder. +/-: heterozygous, +/+: wild type, and -/-: null.

4.6.1.5. Primer sequence

For mouse genotyping primer design and PCR protocol was used as originally provided by Dr Catherine Ledent (Ledent *et al*., 1997) and empirically optimized in our laboratory.

PCR primer nucleotide sequence for forward primer (5'- 3'):

AAG GAA GGG TGA GAA CAG AG for mutant strain detection

CTC CAC CAT GAT GTA CAC CG for normal strain detection

PCR primer nucleotide sequence for reverse primer (5'- 3'):

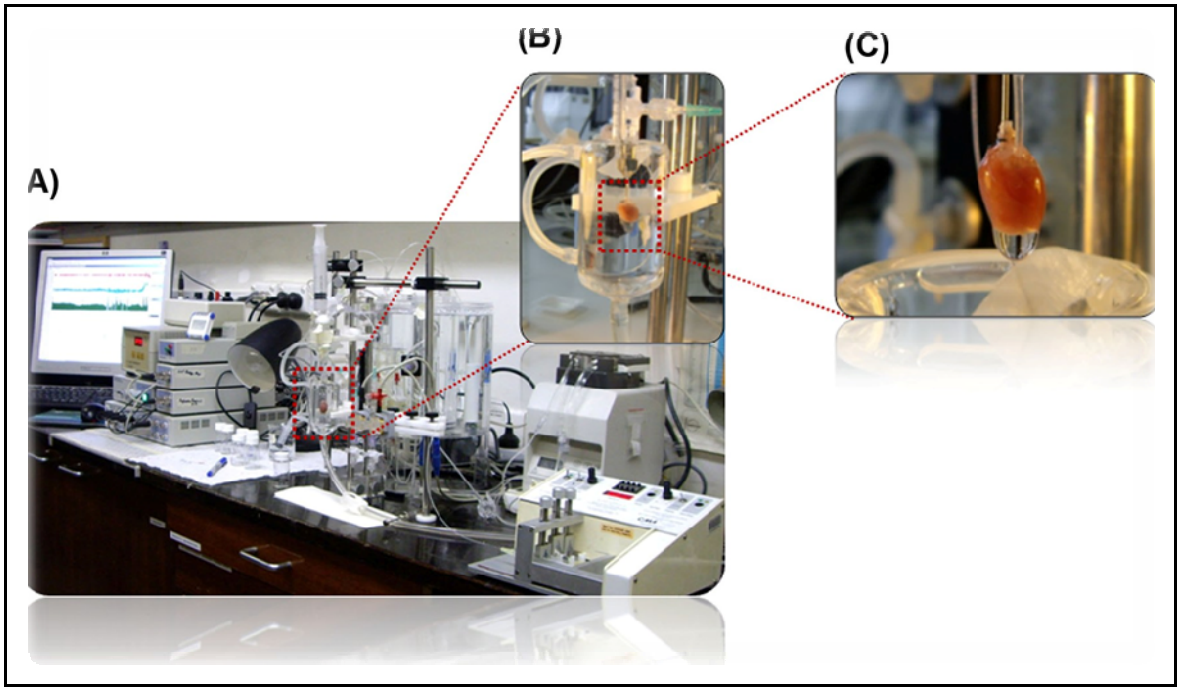
CAT GGT TTC GGG AGA TGC AG

All primers were purchased from Sigma Genosys, Castle Hill, Australia.

4.6.2. Langendorff-perfused isolated mouse heart technique and buffer composition

Mouse isolated heart cannulation in Langendorff- preparation was carried out as previously described (Headrick *et al*., 2001). After anesthesia with sodium pentobarbital (60mg/kg) administered by intraperitonial route, a thoractomy was done in mice and the hearts were rapidly excised into ice cold perfusion buffer. The hearts were retrogradely perfused through the aorta at a constant pressure of 80mmHg with modified Krebs-Henseleit buffer containing (in mM) 120 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 0.5 EDTA, 22 $NaHCO_3$, 11 Glucose, and 2.5 $CaCl_2$. The perfusion buffer was equilibrated with 95% O_2 and 5% CO_2 at 37^0C to maintain the pH at 7.4. The buffer was filtered through an in-line 0.45µM Sterivix-HV filter unit (Millipore, Billerica, MA, USA) to remove micro particulate matters. The left atrium was removed and the left ventricle was vented with polyethylene

tube through the apex for Thebasian drainage, prevented intraventricular pressure development. For measurement of left ventricular diastolic pressure, a fluid-filled balloon made with plastic film inserted in to the left ventricle through the atrium. The balloon was connected to the physiological pressure transducer (MLT844, ADInstruments, Bella Vista, NSW, Australia) and inflated using 500µl glass syringe to obtain ~5mmHg left ventricular diastolic pressure. The heart was submerged in to the heat-jacketed Krebs-Henseleit buffer maintained at 37^0C throughout the experiment (Supplementary Figure 4.2). Perfusion fluid temperature was continuously measured using T-type implantable thermocouple (MLT1401, ADInstruments, Bella Vista, NSW, Australia). Coronary perfusion pressure was measured using physiological pressure transducer (MLT844, ADInstruments, Bella Vista, NSW, Australia) attached to three-way valve immediately above the aortic cannula. Perfusion reservoir and aorta cannula connected to a Minipulse 3 peristaltic pump (Gilson SAS, Villers Le Del, France), was attached to PowerLab (8/30, ADInstruments) via STH Pump controller (ADInstruments, Bella Vista, NSW, Australia). Continuous in-line measurement of coronary perfusion flow was monitored by appropriately calibrated pump, which was recorded on the computer by PowerLab data acquisition system (ADInstruments, Bella Vista, NSW, Australia).



Supplementary Figure 4.2. Langendorff perfused isolated mouse heart setup and physiological recording systems (A), isolated mouse heart submersed in perfusate solution in organ bath (B). Isolated mouse heart is canunlated and a tube connected with fluid filled balloon inserted in the left ventricle (right side) and thermocouple for temperature measurement (left side; C).

4.6.3. Exclusion criteria

Heart were excluded in the events when LVDP was less than 50 mmHg, heart rate less than 200 bpm, coronary flow less than 1 ml/min and greater than 5 ml/min, time delay in aortic cannulation (> 120 sec.), aorta damage during cannulation (detected by fall in coronary perfusion pressure), continuous prolonged arrhythmia during the stabilization period.

4.6.4. Choice of agonist and antagonist concentration and drug administration

The concentration for agonist and antagonist in the present study were selected based on our group's previous findings (Urmaliya *et al*., 2009), in which we demonstrated the concentration response curve for different agonist in rat cardiac cells and selected the most effective concentrations for the present study. Respective agonists and antagonists at final concentration were made in the perfusion buffer in a separated buffer tank in the Langendorff apparatus; however antagonists alone were infused through a microinjection pump (Model No CMA/100, CMA Microdialysis AB, Stockholm, Sweden) at 1% of coronary flow rate with an insertion close to heart at the final concentrations mentioned in the protocol.

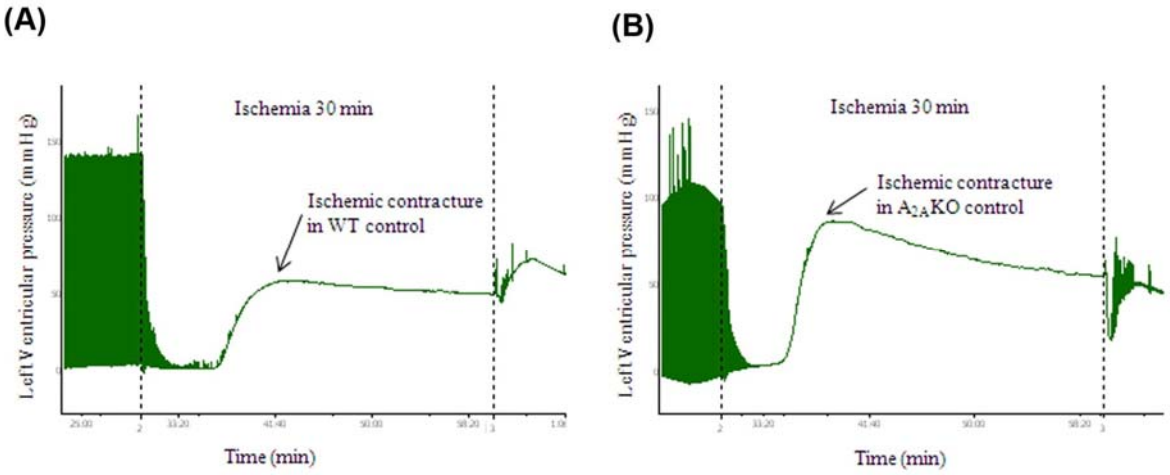
4.6.5. Protein extraction buffers and methods used to detect extracellular signal-regulated kinase (ERK) 1/2 phosphorylation by western blot analysis

RIPA protein lysis buffer composition: 1% Triton X-100. 1% sodium deoxycholate, 0.1% SDS, 0.2mM sodium ortho-vanadate, 0.5mM dithithretol, 250mM NaF, 200mM β-glycerophosphate, 50mM HEPES, 25mM EDTA with 1% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) in 20mM Tris and 150mM Tris and 150 mM NaCl (pH 7.5) and incubated for 20 min at 4^{o}C (Hollande *et al.*, 2001). The resulting homogenate was centrifuged at 5000 g for 20 min followed by the supernatant centrifugation at 10,000 g for 10 min at 4^{o}C. Resulting protein supernatants were collected in fresh tubes and stored in the -80^{o}C until the Western blot experiment.

4.7. Supplementary Results

4.7.1. Ischemic contracture (end diastolic pressure) during ischemic period in WT and A_{2A}KO hearts

We observed greater increase in ischemic contracture (measured as increased EDP) in A_{2A}KO compare to WT hearts during ischemic period. Supplementary Figure 4.3 shows almost double end diastolic pressure in A_{2A}KO hearts (B) during 30 min ischemia compare to WT hearts (A).



Supplementary Figure 4.3. Left ventricular pressure trace in WT (A) and A_{2A}KO mice during ischemia. Higher ischemic contracture was observed in A_{2A}KO mice compared to WT mice during ischemic period.

CHAPTER 5

A novel, highly selective adenosine A_1 receptor partial agonist VCP28 reduces ischemia injury in a cardiac cell line and ischemia-reperfusion injury in isolated rat hearts at concentrations that do not affect heart rate

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5.1. Abstract

The cardioprotective effects of a novel adenosine A_1 receptor partial agonist N^6-(2,2,5,5-tetramethylpyrrolidin-1-yloxyl-3-ylmethyl)adenosine (VCP28) were compared with the selective adenosine A_1 receptor agonist N^6-cyclopentyladenosine (CPA) in a H9c2(2-1) cardiac cell line simulated ischemia (SI) model (12 hrs) and a global ischemia (30 min) reperfusion (60 min) model in isolated rat heart model. H9c2(2-1) cells were treated with CPA and VCP28 at the start of ischemia for entire ischemic duration, while isolated rat hearts were treated at the onset of reperfusion for 15 min. In the H9c2(2-1) cells SI model, CPA and VCP28 (100 nM) significantly ($P<0.05$, n= 5-6) reduced the proportion of nonviable cells (30.88 ± 2.49, 16.17 ± 3.77% of SI group, respectively) and lactate dehydrogenase efflux. In isolated rat hearts, CPA and VCP28 significantly (n= 6-8, $P<0.05$) improved postischemic contractility (dP/dt_{max}, 81.69 ± 10.96, 91.07 ± 19.87 % of baseline, respectively), left ventricular developed pressure, end diastolic pressure and reduced infarct size. The adenosine A_1 receptor antagonist abolished the cardioprotective effects of CPA and VCP28 in SI model and isolated rat hearts. In conclusion, the adenosine A_1 receptor partial agonist VCP28 has equal cardioprotective effects to the full agonist CPA at concentrations that have no effect on heart rate.

5.2. Introduction

Adenosine receptor activation plays a significant role in cardioprotection during ischemia and reperfusion damage (Babbitt *et al.*, 1989; Ely *et al.*, 1992a; Toombs *et al.*, 1992). Endogenous adenosine released during pathophysiological stimuli such as hypoxia, ischemia-reperfusion damage (Ely *et al.,* 1992b; Van Wylen *et al.,* 1992; Headrick 1996) acts through the subtypes A_1, A_{2A}, A_{2B} and A_3 to provide its cardioprotective effects (Fredholm *et al.,* 2001; Linden 2001; Jacobson *et al.,* 2006). Thus, cardioprotection by ischemia preconditioning and attenuation of reperfusion damage by postconditioning together with pharmacological intervention has been of great interest.

Small episodes of discontinuous ischemia prior to a lethal episode of ischemia protect the heart is called "ischemic preconditioning" (Murry *et al.*, 1986; Yellon *et al.*, 2005), and brief infusion of pharmacological agents to mimic this process has been termed "pharmacological preconditioning". Exogenous or endogenous activation of adenosine A_1 receptor during ischemia provides cardioprotection by reduced necrotic cell death in cultured cardiac cells (Stambaugh *et al.,* 1997; Safran *et al.,* 2001; Urmaliya *et al.,* 2009), and reduced infarct size with improved postischemic functional recovery in isolated rat (Lasley *et al.*, 1990), mouse heart (Matherne *et al.,* 1997; Peart *et al.,* 2000; Flood *et al.,* 2002) and *in-vivo* rat heart (Lasley *et al.*, 2007). Reports suggest that activation of the adenosine A_1 receptor by endogenous adenosine and exogenous adenosine A_1 receptor agonists (N^6-cyclohexyl adenosine- CHA; 500 nM) improves postischemic functional recovery in rat and rabbit isolated heart (Headrick 1996). Pre-treatment with selective A_1 adenosine receptor agonists N^6-(phenyl-2R-isopropyl)-adenosine (PIA) and 2-chloro-N^6-

cyclopentyladenosine (CCPA) reduces infarct size and improves functional recovery by ischemic preconditioning mechanism in *in-vivo* rabbit heart (Thornton *et al.*, 1992).

Intermittent episodes of ischemia or a brief infusion of pharmacological agents at the onset of reperfusion is termed as "ischemic or pharmacological postconditioning" (Zhao *et al.,* 2003; Penna *et al.,* 2008). Evidence suggests that adenosine A_1 receptor activation by endogenous adenosine participates in postconditioning cardioprotection as shown by the decrease the contractile recovery in isolated mouse heart after administration of DPCPX during reperfusion (Peart *et al.*, 2000). Targeted deletion of adenosine A_1 receptors reduces ischemic postconditioning cardioprotection as assessed by increased infarct size (Xi *et al.*, 2008), increased postischemic LDH efflux and decreased postischemic function functional recovery (Morrison *et al.*, 2006) in isolated mouse ischemia reperfusion injury. Overexpression of adenosine A_1 receptor, on the other hand, has been shown to reduce contracture and improve contractility in mouse heart during reperfusion (Matherne *et al.*, 1997). Reports suggest that adenosine A_1 receptor activation during reperfusion injury by the exogenous adenosine agonist CHA, CPA (N^6 cyclopentyladenosine), and CCPA give cardioprotection as assessed by increased postischemic functional recovery in isolated rat heart (Lasley *et al.,* 1992; Finegan *et al.,* 1996; Lozza *et al.,* 1997; Lasley *et al.,* 1998) and mouse heart (Butcher *et al.*, 2007), although a number of reports show no effect of A_1 agonists at reperfusion (Thornton *et al.,* 1992; Peart *et al.,* 2002). In many paradigms, across a variety of species, A_1 activation protects the heart from ischemia-reperfusion damage.

In the clinical trials infusion of adenosine itself (Mahaffey *et al.*, 1999; Ross *et al.*, 2005) or an adenosine A_1/A_2 agonist AMP579 (Kopecky *et al.*, 2003) during reperfusion, these agents reduced infarct size when administered to treat acute myocardial infarction, although outcomes of the trails were insignificant because of limited sample size and in the latter loading dose did not achieved protective blood level until 30 min of reperfusion. In the AMISTAD adenosine trial, two doses were used (50 and 70 μg/kg/min), the low dose having no effect on infarct size and the higher dose producing modest reduction in infarct size. The authors of the AMISTAD II trial state that a low dose was used to "reduce the incidence of bradycardia and hypotension" (Ross *et al.*, 2005). A limiting factor in the dose of full A_1 agonist used in these trials was thus the acute cardiac effects, including negative inotropy, chronotropy and dromotropy. Partial A_1 agonists therefore might be of use in this setting, with the possibility of reduced cardiac depression and hemodynamic consequences.

Adenosine receptor agonists are of a great interest as adjunct therapy for ischemic and reperfusion injury to minimize myocardial damage. Recently, we reported a novel potent and selective adenosine A_1 receptor agonist VCP28 (N^6-(2,2,5,5-tetramethylpyrrolidin-1-yloxyl-3-ylmethyl)adenosine), with antioxidant moiety is attached to the N^6 position of the purine ring in VCP28 structure without affecting the adenosine receptor selectivity and cardioprotective effect (Gregg *et al.*, 2007). VCP 28 is 400 fold selective for adenosine A_1 vs. A_3 adenosine receptor and 200 fold more selective for adenosine A_1 vs. A_{2A}, A_{2B} adenosine receptor with high affinity (K_i value 50 nM at A_1 receptor vs. >10,000; 21,200 nM for A_{2A}/A_{2B} or A_3 adenosine receptors, respectively) (Gregg *et al.*, 2007). In the present study we examined the cardioprotective effects of VCP28 during ischemia

(pharmacological preconditioning) using a cardiac cell line simulated ischemia and during reperfusion injury (pharmacological postconditioning) using an isolated rat heart ischemia-reperfusion injury model. We were interested to know whether our novel compound, VCP28, could produce similar cardioprotection to the prototypical full adenosine A_1 agonist N^6-cyclopentyladenosine (CPA) at concentrations that had no effect on heart rate.

5.3. Materials and methods

5.3.1. Cell culture and simulated ischemia

The H9c2(2-1) embryonic rat atrial cell line (American Type Culture Collection-ATCC, Manassas, VA, USA) was used for this study. The cell line was grown in Dulbecco's modified Eagle's medium containing 4 mM L-glutamate, 4.5 g/L glucose, 3.7 g/L sodium bicarbonate, 100 U/ml penicillin and 100 mg /ml streptomycin supplemented with 10% fetal bovine serum (Invitrogen, Mount Waverley, VIC, Australia) in a 5% CO_2 incubator. Cells were used at 60-70 % confluence and plated at the density of $1x10^6$ cells/ml in a 96-well flat-bottomed plate one day prior to assay. Simulated ischemia was induced using conditions developed in our lab and described previously (Gregg *et al.*, 2007; Urmaliya *et al.*, 2009), In short, ischemia was achieved by incubating the cells in hypoxic simulated ischemia (SI) medium at pH 6.4 containing (in mM): 137 NaCl, 3.5 KCl, 0.88 $CaCl_2.2H_2O$, 0.51 $MgSO_4.7H_2O$, 5.55 D-glucose, 4 HEPES, 10 2-deoxy-D-glucose and 20 DL-lactic acid (Sigma, Castle Hill, NSW, Australia) plus 2% fetal bovine serum. Cells were incubated under nitrogen (100% N_2 gas atmosphere) for 12 hrs at 37^oC. The ischemia model induced both apoptosis and necrosis in cultured H9c2(2-1) cells. Under these conditions approximately 40% of all cells stained positive to propidium iodide (which was normalized to 100% SI) indicating necrosis. Fresh simulated ischemia medium was prepared for each experiment. The control group of cells was kept in a normal oxygen incubator (5% CO_2 atmosphere) for the same period of time. In the control group HEPES buffer without 2-deoxy-D-glucose and DL-lactic acid was used.

5.3.2. General experimental protocol for cell culture

H9c2(2-1) cells were incubated in either normal medium (controls) or hypoxic SI medium. Agonists were diluted freshly with PBS to the appropriate concentrations from stock solutions and added at the start of simulated ischemia. When antagonists were used, they were added 20 min before addition of the agonist. Adenosine deaminase; 1U/ml (Maggirwar *et al.*, 1994) when used to remove endogenously released adenosine (convert endogenous adenosine to inosine) from H9c2(2-1) cells during simulated ischemia, was added before addition of either agonists or antagonists. The experimental protocol is summarized in Figure 5.1A.

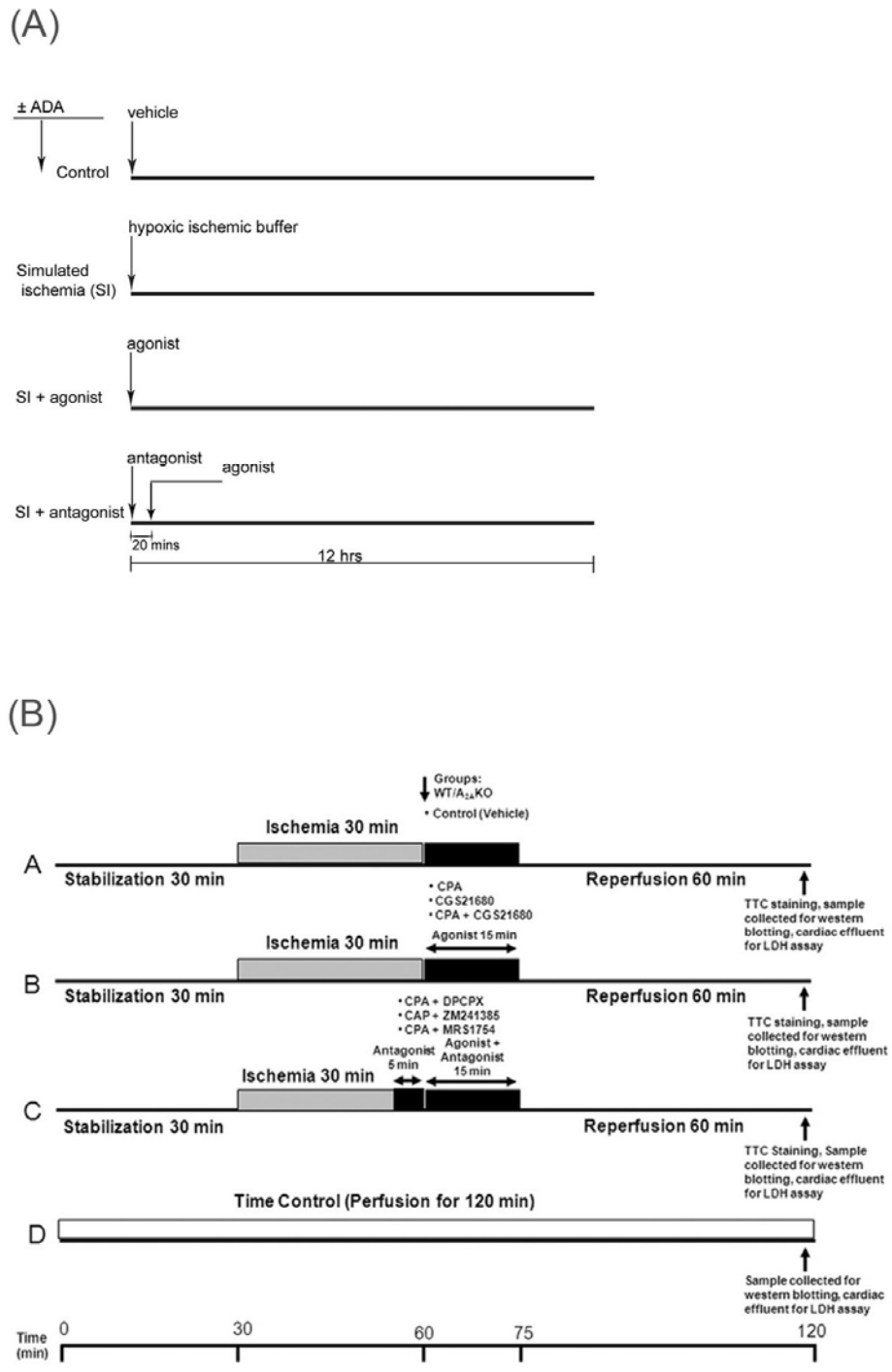


Figure 5.1. Schematic diagram showing the experimental protocol for the H9c2(2-1) cardiac cell line simulated ischemia (SI) model (A) and ischemia reperfusion model in the

isolated rat heart. In the SI model adenosine agonists were used in the absence and presence of adenosine deaminase (ADA) and cells were exposed to the simulated ischemic condition for 12 hrs. In the isolated rat heart global ischemia (30 min) and reperfusion (60 min) model, agonist and antagonist were added at the onset of reperfusion.

5.3.3. Experimental groups for simulated ischemia assay in cell culture

Experiments were divided into following groups-

(A) Effect of adenosine A_1 receptor activation during ischemic conditioning of H9c2(2-1) cells- (i) control, (ii) simulated ischemia (SI), (iii) SI + CPA or VCP28 (10^{-8} - 10^{-6}M; selective A_1 antagonist and a novel A_1 agonist synthesized in our faculty by the department of Medicinal Chemistry, respectively), (iv) SI + agonists (CPA or VCP28) + antagonist (DPCPX 10^{-6} M; adenosine A_1 receptor selective).

(B) Effect of A_1 receptor activation in the presence and absence of endogenous adenosine during simulated ischemia- (i) control, (ii) SI + ADA, (iii) SI + ADA + CPA or VCP28 (10^{-8} – 10^{-6} M), (iv) SI + ADA + agonist (CPA or VCP28) + antagonist (DPCPX; 10^{-6} M).

5.3.4. Cell viability (Propidium Iodide) Assay and Imaging of H9c2(2-1) cells

For detection of non-viable cells (necrosis) in all groups**,** a propidium iodide assay was used. At the end of the period of simulated ischemia, 5μM propidium iodide (PI; Sigma, NSW, Australia) was added to each well and incubated for 15 min in the dark. After 15 min images were taken using an inverted fluorescence microscope connected to a SPOT RT camera (Nikon Eclipse TE2000U, Nikon Instruments, Japan) at 535 nm excitation

wavelength achieved by DG-4 light box (Shutter Instruments, USA) and 617 nm emission filters. Duplicate wells were used for each experiment, and each experiment was repeated three times. From each well 4 images were taken, and propidium iodide positive cells were quantified with using Scion Image Alpha 4.0.3.2 software (NIH Image, National Institute of Health, USA) then normalized to the SI treatment group (i.e. necrosis was expressed as % cell death with respect to the control SI group using this model).

5.3.5. Lactate dehydrogenase (LDH) release from cardiac effluent

Ischemic injury-induced cellular damage was evaluated in H9c2(2-1) cells after simulated ischemia model by the measurement of LDH release from cell supernatant collected at the end of ischemia protocol. LDH release was measured by using a commercial kit; CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madosin, WI, USA) as per manufacturer guidelines. CytoTox® 96 Non-Radioactive Cytotoxicity Assay is a colorimetric assay quantitatively measures LDH released upon cell lysis or damage. LDH released in a mouse cardiac effluent during ischemia reperfusion injury was measured with a 30 min coupled enzyme assay, which result in the conversion of a tetrazolium salt into a red formazan product. The amount of color formed is proportional to the percentage damage of cardiac cells, which was expressed as percentage cytotoxicity.

5.3.6. Animals

All animal experiments were conducted follows the requirements from the Prevention of Cruelty to Animals Regulations, 2008, under the guidelines of the Australian National Health and Medical Research Council Code of Practice for the Care and Use of Animals for

Scientific Purposes in Australia. The adult male rats (Asmu:SD strain) used in the present study were obtained from Monash Animal Services, Monash University. Rats were maintained in-house at the Monash Institute of Pharmaceutical Sciences animal house at 22 ºC with a constant 12 hour light/12 hour dark cycle. Animals had free access to rat chow and water *ad libitum.* The experimental protocols were formally approved by the Monash University Standing Committee of Animal Ethics in Animal Experimentation, Faculty of Pharmacy and Pharmaceutical Sciences, Monash University, Parkville campus, Australia (# RPW 2006/01).

5.3.7. Langendorff-perfused isolated rat heart model

Langendorff-perfused isolated heart preparation in rat was carried out as previously described (Rose'Meyer *et al.*, 2003). Male rats (250-300 gm) were anesthetized with sodium pentobarbital (60 mg/kg) administered by intraperitonial route, a thoractomy was done and the hearts were rapidly excised into ice cold perfusion buffer. The hearts were retrogradely perfused through the aorta at a constant pressure of 80mmHg with modified Krebs-Henseleit buffer containing (in mM) 120 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 0.5 EDTA, 22 $NaHCO_3$, 11 Glucose, and 2.5 $CaCl_2$. The perfusion buffer was equilibrated with 95% O_2 and 5% CO_2 at 37^0C to maintain the pH at 7.4. The buffer was filtered through an in-line sterile 0.45µM Sterivix-HV filter unit (Millipore, Billerica, MA, USA) to remove micro particulate matters. The left atrium was removed and the left ventricle was vented with polyethylene tube through the apex for Thebasian drainage, prevented intraventricular pressure development. For measurement of left ventricular diastolic pressure, a fluid-filled latex balloon (#170403, size 3; ADInstruments, Bella Vista, NSW,

Australia) inserted in to the left ventricle through the atrium. The balloon was connected to the physiological pressure transducer (MLT844, ADInstruments, Bella Vista, NSW, Australia) and inflated using 500µl glass syringe to obtain approximately 5mmHg left ventricular diastolic pressure. The heart was submerged in to the heat-jacketed Krebs-Henseleit buffer maintained at 37^0C throughout the experiment. Perfusion fluid temperature was continuously measured using T-type implantable thermocouple (MLT1401, ADInstruments, Bella Vista, NSW, Australia). Coronary perfusion pressure was measured using physiological pressure transducer (MLT844, ADInstruments, Bella Vista, NSW, Australia) attached to three-way valve immediately above the aortic cannula. Perfusion reservoir and aorta cannula connected to a Minipulse 3 peristaltic pump (Gilson SAS, Villers Le Del, France), was attached to PowerLab (8/30, ADInstruments) via STH Pump controller (ADInstruments, Bella Vista, NSW, Australia). Continuous in-line measurement of coronary perfusion flow was monitored by appropriately calibrated pump, which was recorded, along with the coronary perfusion pressure, on the computer by PowerLab data acquisition system (ADInstruments, Bella Vista, NSW, Australia). The left ventricular pressure signals acquired continuously using a PowerLab and digitally processed by using ChartPro version 5.5.6 software (ADInstruments) to measure left ventricular systolic and diastolic pressure, heart rate, dP/dt_{max}, and dP/dt_{min}. Difference of systolic to diastolic pressures was used to obtain Left ventricular developed pressure (LVDP).

5.3.8. Exclusion criteria

Heart were excluded in the events when left ventricular developed pressure was less than 50 mmHg, heart rate less than 200 beats per minute, coronary flow less than 3 ml/min and

greater than 12 ml/min, time delay in aortic cannulation (> 120 sec.), aorta damage during cannulation (detected by fall in coronary perfusion pressure), continuous prolonged arrhythmia during the stabilization period.

5.3.9. Global ischemia-reperfusion technique

All hearts were equilibrated for 30 min to allow for stabilization before ischemia reperfusion protocol. After stabilization baseline functional measurements were acquired immediately before hearts were subjected to 30 min of no-flow normothermic global ischemia and 60 min of reperfusion (I-R). Ischemia was attained by completely stopping the perfusate inflow and immersing the heart in perfusate buffer which was saturated with 95% N_2 + 5% O_2 in the organ bath. After no-flow global ischemia, reperfusion was achieved by opening the perfusion in-line flow. To examine the adenosine A_1 receptor-mediated cardioprotective effect on functional recovery, infarct size reduction using a selective and a novel adenosine A_1 receptor agonist, hearts were perfused with buffers containing the indicated agonist and antagonist concentrations for 15 min at the start of reperfusion. When antagonists were used, they were perfused for 5 min before addition with agonist for 15 min. In the present study 30 min of global ischemia and 60 min of reperfusion protocol was selected based on the empirical studies performed in our laboratory (data not shown) for different time points to observe significant contracture during ischemia, which would not give complete necrosis to the isolated rat heart.

5.3.10. Effect of adenosine A_1 agonist and a novel A_1 agonist on isolated rat heart rate

Separate experiments were performed to establish the potency and efficacy of adenosine A_1 agonist CPA and a novel adenosine A_1 agonist VCP28 on adenosine A_1 receptor-mediated heart rate reduction, concentration response curve were obtained on Langendorff perfused isolated rat heart without ischemia and reperfusion protocol.

5.3.11. Experimental groups and protocol for ischemia reperfusion injury in isolated rat heart

Animals were divided in to the following experimental groups (Figure 5.1 B). Group 1: Control- rat hearts were exposed to global ischemia (30 min) and reperfusion (60 min); Group 2: CPA (10^{-7} M)- rat hearts were exposed to I-R with CPA (10^{-7} M) perfusion at 0- 15 min reperfusion; Group 3: VCP28 (10^{-7} M)- rat hearts were exposed to I-R with VCP28 (10^{-7} M) perfusion at 0- 15 min of reperfusion; Group 4: CPA (10^{-7} M) + DPCPX (10^{-6} M)- rat hearts were exposed to I-R with DPCPX (10^{-6} M) perfusion for 5 min before reperfusion and combination with CPA (10^{-7} M) at 0- 15 min of reperfusion; Group 5: VCP28 (10^{-7} M) + DPCPX (10^{-6} M)- rat hearts were exposed to I-R with DPCPX (10^{-6} M) perfusion for 5 min before reperfusion and combination with VCP28 (10^{-7} M) at 0- 15 min of reperfusion. The concentration for agonist and antagonist in the present study were selected based on our group's previous findings (Urmaliya *et al.*, 2009) , in which we demonstrated the concentration response curve for CPA and DPCPX in rat cardiac cells. We established the potency and efficacy of CPA and VCP28 by measuring concentration response curve for the A_1 receptor-mediated heart rate reduction in isolated rat heart in the present study and selected the most effective concentrations. Respective agonists and antagonists at final

concentration were made in the perfusion buffer in a separated buffer tank in the Langendorff apparatus; however antagonists alone were infused through a microinjection pump (Model No CMA/100, CMA Microdialysis AB, Stockholm, Sweden) at 1% of coronary flow rate with an insertion close to heart at the final concentrations mentioned in the protocol.

5.3.12. Infarct size measurement

At the end of the experiment, a subset of hearts were quickly removed, weighed, wrapped in a plastic film and placed at -20^{0}C for 5-6 hrs. The frozen hearts were sectioned into 7-8 transverse slices of approximately equal thickness of 1.5 mm. They were stained by incubation in 1% 2, 3, 5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich, St Louis, MO, USA) for 15 min at 37^{0}C. After fixing in 10% formalin saline for overnight, heart sections were scanned using a flatbed scanner (K10291, Canon Scan LiDE 600F). The area of infarction and risk zone was measured using ImageJ 1.41o software (National Institute of Health, USA). The risk area was calculated as total ventricular area minus cavities. Infarct size is expressed as a percentage of the risk area.

5.3.13. Drugs and Preparation of Stock Solutions

CPA, DPCPX, and adenosine deaminase (adenosine deaminase from bovine spleen Type X, buffered aqueous glycerol solution, 130 units/mg protein) were purchased from Sigma-Aldrich (Castle Hills, NSW, Australia). All other reagents and chemicals were purchased from Sigma-Aldrich (Castle Hills, NSW, Australia). The novel adenosine A_1 receptor agonist VCP28 (Figure 5.2A) was synthesized in house as described previously (Gregg *et*

al., 2007). Stock solutions were prepared in dimethyl sylfoxide (DMSO) for all agonists and antagonists containing no more than 0.1% DMSO and stored at -20^{0}C. At the time of experiment stock solutions were freshly diluted in perfusion buffer to make the required concentration.

5.3.14. Statistical analysis

The data were presented as a mean ± SEM. In cell culture simulated ischemia experiments cell viability data and LDH release were analyzed by one way ANOVA using Bonferroni's post-hoc test for multiple comparisons. For the cell viability assay non-viable cells from SI group were normalized to 100%. Cardiac functional data at baseline and after ischemia-reperfusion in isolated rat hearts groups were analyzed by two-way ANOVA followed by Bonferroni's post hoc test. Cardiac functional data at 60 min of reperfusion and infarct size data from isolated rat hearts were analyzed by one-way ANOVA followed by Bonferroni's post hoc test. $P < 0.05$ was considered statistically significant.

5.4. Results

5.4.1. Baseline functional data in isolated rat heart

Average heart weight to body weight ratios and heart rate at the end of stabilisation were not different between various groups in isolated rat heart preparation (Table 5.1).

Table 5.1. Baseline functional data in isolated rat heart subjected to global ischemia (30 min) followed by reperfusion (60 min)

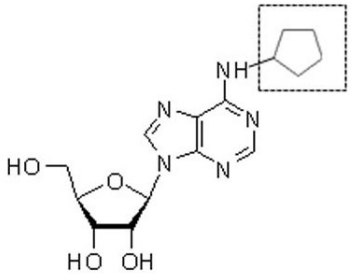
Groups	Heart to body weight ratio (%)	Heart rate (beats per min)
Control	0.53 ± 0.02	244.3 ± 13.06
CPA (100 nM)	0.54 ± 0.03	251.6 ± 4.61
VCP28 (100 nM)	0.56 ± 0.02	266.6 ± 9.45
CPA (100 nM) + DPCPX (1 μM)	0.51 ± 0.01	249.0 ± 4.37
VCP28 (100 nM) + DPCPX (1 μM)	0.53 ± 0.01	248.0 ± 5.17

5.4.2. Concentration response curve of CPA and VCP28 on adenosine A_1 receptor-mediated heart rate and dP/dt_{max} reduction in isolated rat heart

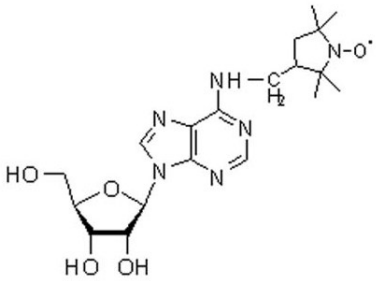
The adenosine A_1 receptor agonist CPA and a novel adenosine A_1 receptor agonist VCP28 produced concentration dependent decreases in heart rate (10^{-11} – 10^{-4} M; Figure 5.2C). CPA demonstrated higher potency than VCP28 with pEC50 values of 7.12 ± 0.16 and 5.16 ± 0.34, respectively. The maximal efficacy of CPA and a novel adenosine A_1 agonist

VCP28 was measured at a dose of 10^{-4} M; the maximum decrease in isolated rat heart rate was significantly higher for CPA than VCP28 (heart rate decreased to 19.13 ± 3.0 and 47.40 ± 6.48 % of baseline, n= 6-8), respectively. CPA and VCP28-mediated reductions in heart rate were blocked in the presence of the adenosine A_1 receptor antagonist DPCPX (10^{-6} M; data not shown). CPA and VCP28 produced modest concentration-dependent decreases in contractility (10^{-11} to 10^{-4} M; Fig. 5.2 D). In this case, VCP28 showed no significant difference in potency to CPA (pEC50 values 9.3% ± 0.90% for VCP28 and 8.1% ± 0.96% for CPA, n = 4–6, P>0.05), and no significant difference in maximal efficacy (n = 4–6, P>0.05).

(A)



CPA



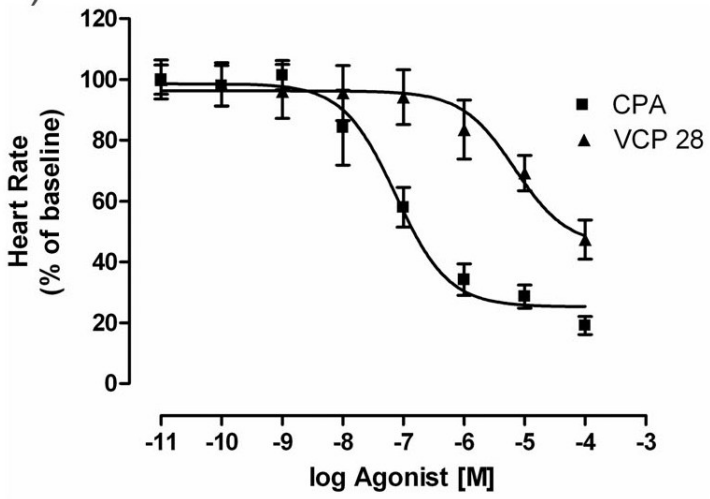
VCP28

(B)

Agonist	K_i value for AR (nM)*				Reference
	Hu A_1 AR	Hu A_{2A} AR	Hu A_{2B} AR	Hu A_3 AR	
CPA	2.3	794	18,600#	72	Jacobson and Gao 2006
VCP28	50	>10,000	>10,000	21,200	Gregg *et al* 2007

*Competitive binding assay at recombinant human A_1, A_{2A}, A_{2B} and A_3 adenosine receptors.
#Cyclic AMP functional assay

(C)



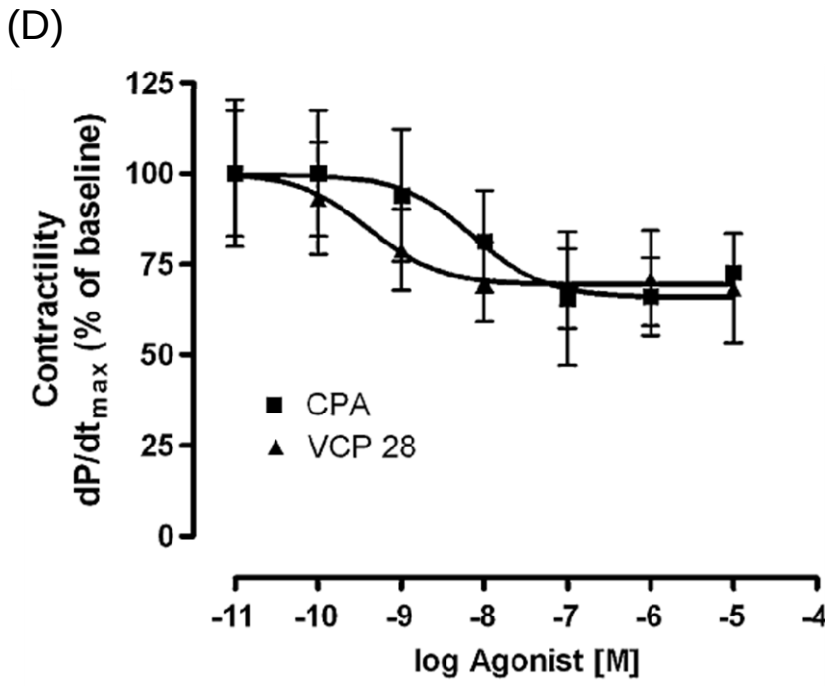


Figure 5.2. (A) Chemical structure of CPA and VCP28, (B) affinity (Ki value in nM) data for CPA and VCP28 in human adenosine A_1, A_{2A}, A_{2B} and A_3 receptor using competitive binding assay, Concentration response curve for CPA and VCP28 effects on heart rate (C) and contractility dP/dt_{max} (D) in Langendorff perfused isolated rat heart. The heart rate data were normalized to baseline values and expressed as percentage of baseline (mean ± SEM, n=6-8).

5.4.3. Effects of CPA and VCP28 on cell viability during SI in the presence and absence of endogenous adenosine

Incubation of H9c2(2-1) cells in SI buffer for 12 hrs significantly increased the proportion of nonviable cells compared to HEPES buffer control (48.42 ± 8.11% cells were PI positive in SI buffer vs. 7.36 ± 1.18% PI positive cells in control, n= 5-6, $P<0.05$, Figure 5.3). The SI group was assigned a value of 100% and other treatment groups were expressed as % normalized to the SI group. CPA and VCP28 (10^{-7} M) significantly reduced the proportion

of nonviable cells (30.88 ± 2.49 and 16.17 ± 3.77% with reference to the SI group, respectively, $P<0.05$, Figure 5.3A, C). Removal of endogenous adenosine by addition of ADA in SI buffer significantly increased the number of nonviable cells. In the presence of ADA, the protective effect of CPA and VCP28 was reduced (66.93 ± 3.75 and 37.65 ± 5.20% with reference to the SI group, respectively, Figure 5.3 B, D). Cardioprotective effects of CPA and VCP28 were significantly reversed by the adenosine A_1 antagonist DPCPX in the absence and presence of ADA (53.93 ± 4.32, 104.1 ± 7.15 and 69.57 ± 8.0, 90.46 ± 11.98% of nonviable cells with reference to the SI group, respectively, $P<0.05$, Figure 5.3 A- D).

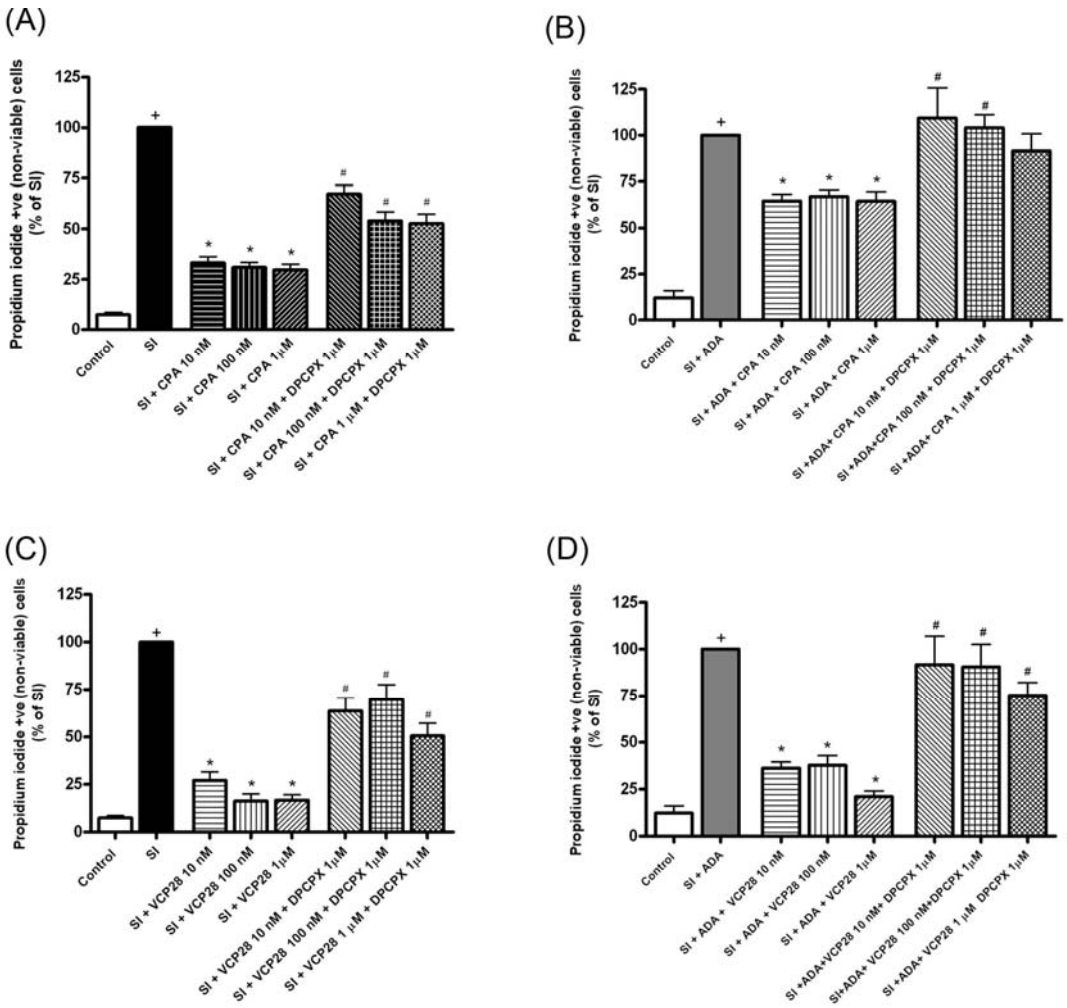


Figure 5.3. Effect of adenosine A_1 receptor activation in the presence of CPA and VCP28 on cell survival during SI. Cardiac cells were incubated in the presence of CPA or VCP28 with SI buffer (A, C, respectively) or with ADA with or without DPCPX (B, D, respectively). Cardiac cells were stained with propidium iodide (PI) to measure nonviable cells (mean ± SEM, n=5-6, $P<0.05$, $^{+}$significantly different to control group, *significantly different to SI group and $^{\#}$significantly different to agonist treated groups).

5.4.4. Effect of CPA and VCP28 on LDH release during SI in H9c2(2-1) cells in the presence and absence of endogenous adenosine

LDH release significantly increased in the SI and SI + ADA groups compared to the non-ischemic control group (39.97 ± 2.20 and 46.62 ± 2.13 vs. 3.01 ± 0.76 and 4.37 ± 0.68% cytotoxicity, respectively, n= 5-6, $P<0.05$, Figure 5.4 A, B). Both CPA and VCP28 produced concentration-dependent decreases in LDH release (10^{-6} M; 24.06 ± 1.71 and 30.80 ± 0.87 vs. 39.97 ± 2.20% cytotoxicity, respectively, $P<0.05$, Figure 5.4 A, C). In absence of ADA, CPA and VCP28-mediated LDH release was significantly blocked in the presence of DPCPX (10^{-6} M). However in the presence of ADA only CPA-mediated, but not the VCP28-mediated reduction in LDH release was abolished by the DPCPX (Figure 5.4 B, D).

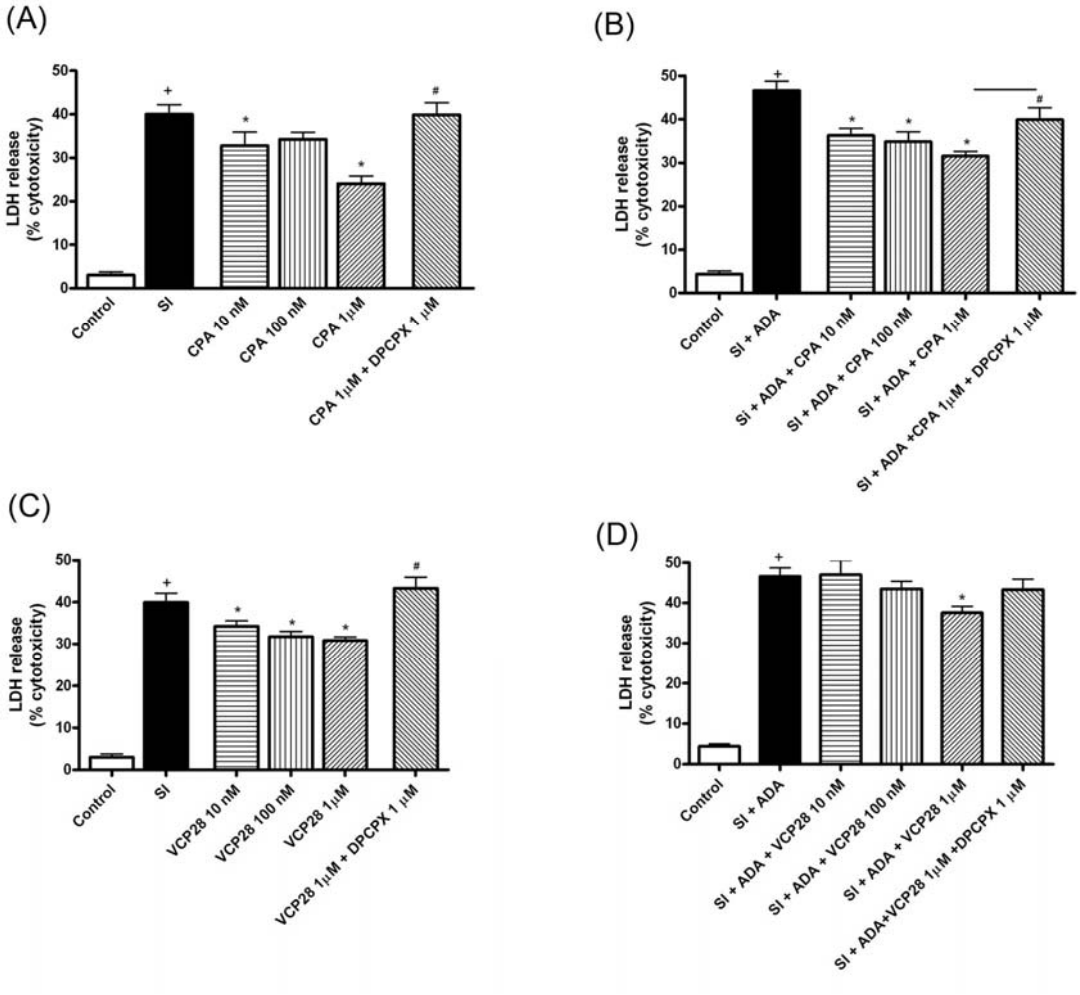


Figure 5.4. LDH release from cardiac cell supernatant after 12 hrs of simulated ischemia. Cells were treated with CPA or VCP28 in the presence (A, C, respectively) and absence of ADA (B, D, respectively). The LDH samples were collected at the end of SI protocol and data represented as percentage cytotoxicity (mean ± SEM, n=5-6, $P<0.05$, $^{+}$significantly different to control group, *significantly different to SI group and $^{\#}$significantly different to agonist treated groups).

5.4.5. Effects of CPA and VCP28 on postischemic functional recovery in rat I-R protocol

Post-ischemic contractility (dP/dt_{max}) and left ventricular developed pressure (LVDP) were significantly reduced at the end of reperfusion period in ischemic control isolated rat hearts (15.85 ± 7.46 and 22.63 ± 14.80% of baseline, respectively, n= 6-8, $P<0.05$, Figure 5.5 A-D). Contractility and LVDP was significantly improved in the presence of CPA and VCP28 (10^{-7} M) compared to ischemic control at the end of reperfusion period (dP/dt_{max}- 81.69 ± 10.96 and 91.07 ± 19.87 vs. 15.85 ± 7.46% of baseline, respectively; LVDP- 69.24 ± 13.13 and 96.71 ± 20.37 vs. 22.63 ± 14.80% of baseline, respectively, $P<0.05$, Figure 5.5 B, D). CPA and VCP28-mediated increases in postischemic contractility and LVDP recovery were abolished in the presence of DPCPX (10^{-6} M; dP/dt_{max}- 16.50 ± 7.28 and 34.14 ± 13.96% of baseline, respectively; LVDP- 24.74 ± 10.03 and 12.20 ± 3.02% of baseline, respectively, $P<0.05$, Figure 5.5 B, D).

Increase in the end diastolic pressure (EDP) after the onset of ischemia is a damaging consequence of this pathology known as ischemic contracture. After 10 min of ischemia a significant increase in the EDP was observed in ischemic control isolated rat hearts (37.81 ± 4.53 mmHg, Figure 5.5 E). CPA treatment but not VCP28 treatment significantly reduced the postischemic contracture compared to ischemic control at a single time point at the end of reperfusion (12.76 ± 2.64 vs. 29.60 ± 3.23 mmHg, n= 6-8, $P<0.05$, Figure 5.5 F). An apparent VCP28-mediated reduction in postischemic contracture was statistically not significant (19.52 ± 6.33 vs. 29.60 ± 3.23 mmHg, $P>0.05$, Figure 5.5 F). CPA-mediated

effects on ischemic contracture reduction was significantly abolished in the presence of DPCPX ($P<0.05$, Figure 5.5 F).

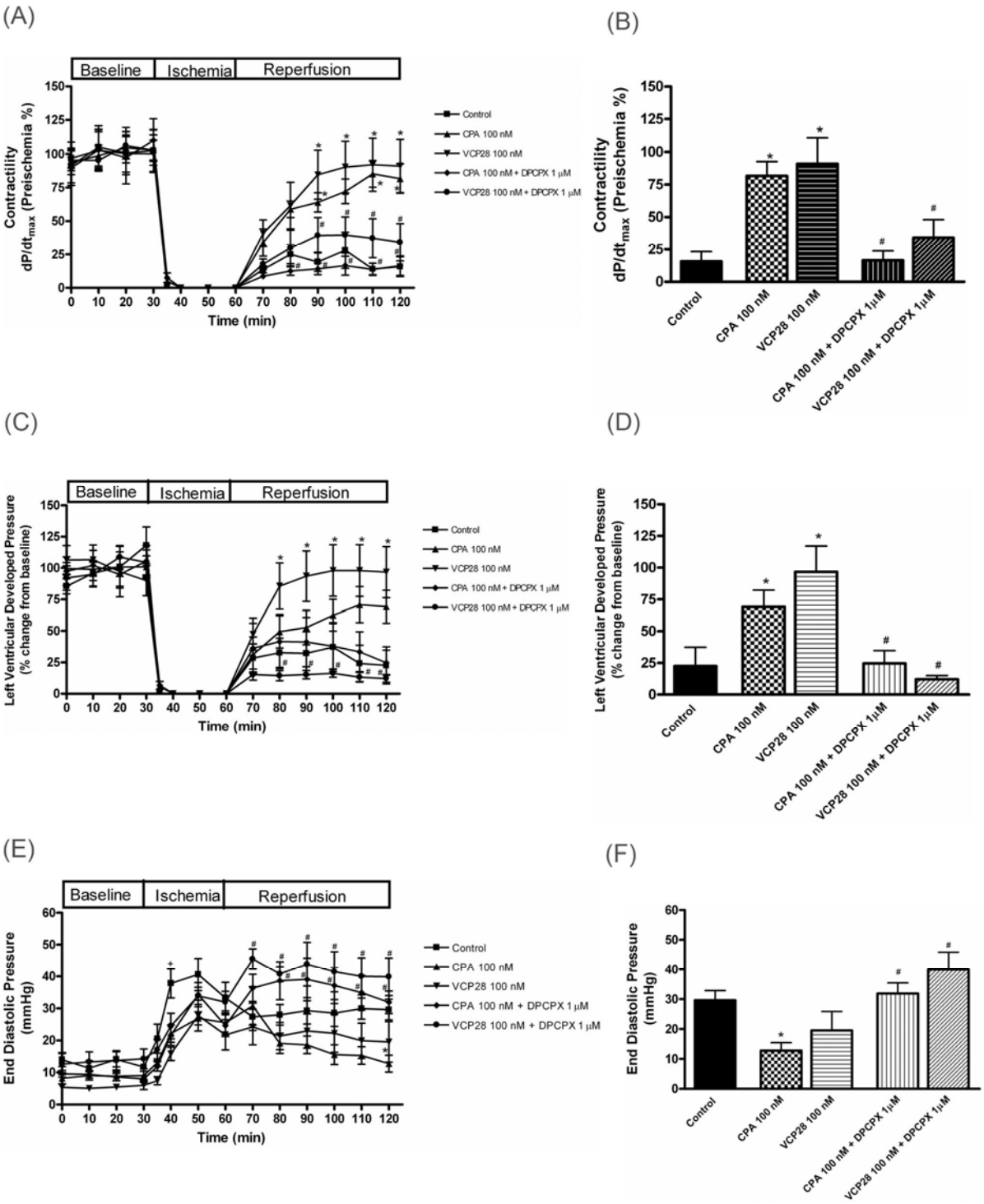


Figure 5.5. Contractility (dP/dt_{max}; A),left ventricular developed pressure (LVDP; C) and

end diastolic pressure (EDP; E) during global ischemia and reperfusion in the presence of CPA and VCP28 (10^{-7} M). B, D and F show dP/dt_{max}, LVDP and EDP, respectively at a single time point at the end of reperfusion in the presence of CPA, VCP28 alone or in combination with DPCPX (10^{-6} M). Contractility and LVDP data were normalized to baseline values and expressed as pre ischemic percentage. EDP data is expressed as mmHg. Values were expressed as mean ± SEM. (n=6-8, $P<0.05$, $^{+}$significantly different to control group, *significantly different to ischemic control and $^{\#}$significantly different to agonist treated groups).

5.4.6. Effect of adenosine A_1 receptor agonist on postischemic infarct size

Postischemic infarct size measured at the end of reperfusion was significantly reduced in the presence of CPA and VCP28 (10^{-7} M) compared to the ischemic control isolated rat heart (17.39 ± 2.58 and 14.88 ± 0.95 vs. 35.03 ± 4.24% area at risk, respectively, n= 6-8, $P<0.05$, Figure 5.6). CPA and VCP28-mediated infarct size reductions were abolished in the presence of the adenosine A_1 antagonist DPCPX (10^{-6} M; 30.74 ± 2.08 and 29.13 ± 6.84% area at risk, respectively, $P<0.05$, Figure 5.6).

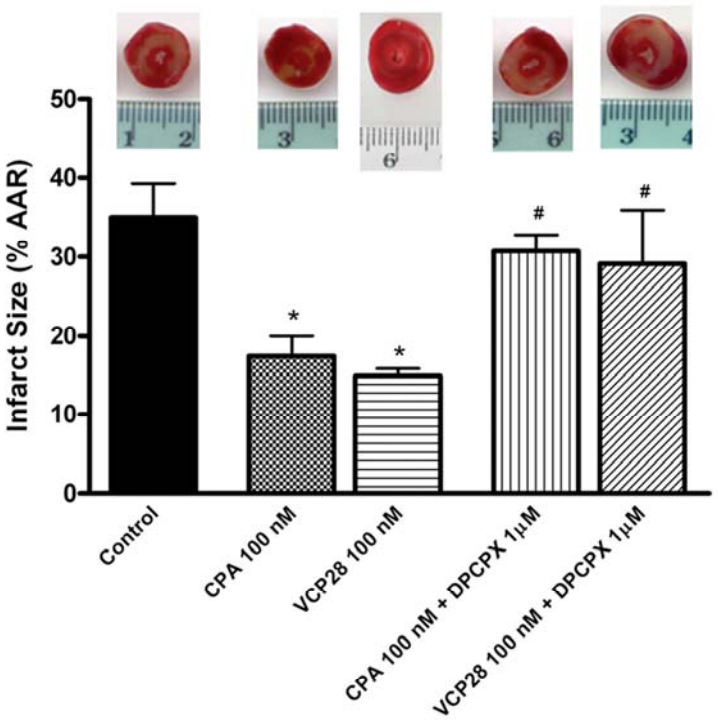


Figure 5.6. Myocardial infarct size measurement in isolated rat hearts after global ischemia and reperfusion injury in the presence of CPA or VCP28 (10^{-7} M) alone or in combination with DPCPX (10^{-6} M). The infarct size data expressed as percentage area at risk (mean ± SEM, n=6-8, $P<0.05$, $^{+}$significantly different to treated group, *significantly different to ischemic control and $^{\#}$significantly different to agonist treated groups).

5.5. Discussion

In the present study we demonstrated that a novel adenosine A_1 partial agonist VCP28 reduces ischemic cell death when administered at the onset of ischemia in a rat cardiac cell ischemia model, and that VCP28 administration during reperfusion injury can significantly reduce infarct size and improve post ischemic functional recovery as assessed by improved contractility and left ventricular developed pressure in the isolated rat heart. We show that a concentration of VCP28 that has no effect on heart rate and is likely therefore to be very well tolerated in humans has profound cardioprotective effects.

5.5.1. VCP28 is a weak partial A_1 agonist in terms of bradycardia and a potent full agonist in terms of negative inotropy in nonischemic rat hearts

VCP 28 has high affinity for adenosine A_1 receptors (Ki = 50 nM) and more than 400 times selectivity for adenosine A_1 versus A_3 (Figure 5.2B). The structure of VCP28 is closely related to CPA with modification at the N^6 position in the purine ring (Figure 5.2A). VCP28 showed approximately 100-fold lower potency as a negative chronotrope compared to CPA (pEC50 value 5.16 for VCP28 and 7.12 for CPA). Also, the maximum efficacy of VCP28 was approximately 1.5 times less than CPA for this parameter (Figure 5.2C). At the concentration we used in the ischemia protocols, VCP28 has little or no effect on heart rate in isolated rat hearts.

5.5.2. Cardioprotection by VCP28 in SI model- Cardioprotection equal in magnitude of that CPA

In the current study A_1 agonists were cardioprotective when given during an ischemic period, in concordance with a large body of previous work in rat cardiac cells (Safran *et al.,* 2001; Gregg *et al.,* 2007; Urmaliya *et al.,* 2009). In the present study, we observed comparable CPA-mediated cardioprotection in a similar cardiac cell ischemia model to that reported previously (Urmaliya *et al.*, 2009). The cardioprotection provided by VCP28 during ischemia was A_1-receptor mediated, since it was abolished in the presence of adenosine A_1 antagonist DPCPX. In the present study we observed that administration of the adenosine A_1 agonist VCP28 and CPA during ischemia period significantly reduced necrotic cell death and reduced LDH release in a cardiac cell ischemia model. Interestingly, addition of adenosine deaminase (ADA) increased the cell death and LDH release in our cardiac cell ischemia model, and diminished the protective effects of VCP28 and CPA. Therefore, the present data shows that endogenous adenosine plays an important role in the cardioprotection conferred by exogenous adenosine A_1 receptor agonists in this cardiac cell ischemia model, in agreement with our previous finding (Urmaliya *et al.*, 2009), but extending the observation to the partial agonist VCP28.

5.5.3. Cardioprotection by VCP28 given for 15 minutes at reperfusion in an isolated rat heart model of I-R

We observed that VCP28 and CPA when administered during reperfusion reduce infarct size and improved postischemic functional recovery by increased contractility and left ventricular pressure in isolated rat heart. CPA treatment during reperfusion reduced postischemic contracture, whilst for VCP28 there was an apparent trend towards reduced EDP, but this did not reach statistical significance. Cardioprotective effects of VCP28 and

CPA were again A_1-mediated in this model, as indicated by reversal using the adenosine A_1 antagonist DPCPX. Our finding that adenosine A_1 receptor activation by exogenous adenosine A_1 agonist during reperfusion injury reduces infarct size and improve postischemic functional recovery is in agreement with other studies in isolated rat heart (Lasley *et al.,* 1992; Finegan *et al.,* 1996; Lozza *et al.,* 1997) and a study in mouse heart (Butcher *et al.*, 2007) , but in contrast with others from various species; mouse heart (Peart *et al.*, 2002), *in-vivo* rat heart (Kin *et al.,* 2005a) and *in-vivo* rabbit heart(Baxter *et al.*, 2000). There are many differences between the studies; agents used, ischemia reperfusion protocols and species differences are all likely causes of contrasting data. What is clear is that a number of well controlled studies have shown A_1-mediated protection when agonists are given at reperfusion.

Of note, the partial agonist VCP28 produced cardioprotection equal to or greater than the full agonist CPA, at concentrations of VCP28 (10^{-7} M) that had modest or no effect on heart rate. To the best of our knowledge, this is the first evidence for partial agonists to activate cardioprotective signalling pathways with greater efficacy and potency than pathways mediating acute cardiac effects. This may have implications for fields other than ischemia; the use of adenosine A_1 agonists as anti-arrhythmic agents is restricted by unwanted cardiovascular side effects such as AV block, bradycardia, atrial fibrillation (Elzein *et al.*, 2008). Indeed, the adenosine A_1 partial agonist CVT-2759 slows AV node conduction and heart rate without AV conduction block and bradycardic effect in isolated guinea pig heart (Wu *et al.*, 2001). Adenosine A_1 agonists exert negative chronotropic and dromotropic effect by slowing sino-atrial (SA) and atrio-ventricular (AV) nodal

conduction, respectively (Wang *et al.,* 1994; Belardinelli *et al.,* 1995) and reduce heart rate by their action on inward rectifying potassium current, inhibition of catecholamine generated pacemaker current (I_F) and L-type Ca^{2+} current, which prolong the AV node refractory period, reduce AV node action potential, and slow SA pacemaker rate (Wang *et al.,* 1994; Belardinelli *et al.,* 1995; Elzein *et al.,* 2008). The other advantage of partial agonists in this context is the potential to avoid desensitization of the receptor because of overstimulation (Elzein *et al.*, 2008).

The signalling pathways involved in A_1-mediated cardioprotection are complex (see review by Headrick *et al.*, (2009) and Peart *et al* .(2007)); the bulk of the experimental evidence suggests that phosphorylation of ERK1/2 (Germack *et al.,* 2004; Germack *et al.,* 2005) and opening of K_{ATP} channels are key steps (Grover *et al.,* 1992; Baxter *et al.,* 1999; Headrick *et al.,* 2000). It appears from our work that VCP28 can cause maximal activation of these protective pathways to elicit adenosine A_1-mediated cardioprotection in cardiomyocytes, whilst higher EC_{50} values for the negative chronotropic effect than CPA (6.84×10^{-6} and 7.47×10^{-8}) suggests that VCP28-occupied A_1 receptors affecting pacemaker potentials signal more weakly. Whilst it is difficult to study differential second messenger activation across different native cell types, the underlying mechanisms are of significant interest.

5.5.4. Conclusions

In the present study we demonstrated that administration of a novel adenosine A_1 receptor agonist VCP28 during ischemia in a cardiac cell ischemia model and during reperfusion in the isolated rat heart model is profoundly cardioprotective. Furthermore, we show that the

cardioprotective effect of VCP28 occurs at concentrations that have minimal bradycardic effect in rat hearts. This compound is of interest as a potential therapy for ischemia-reperfusion injury.

CHAPTER 6

General Discussion

6.1. Analysis of the inventive research presented in this thesis

The work in the present thesis explored the cardioprotective effects of adenosine A_1, A_{2A}, A_{2B} and A_3 receptor activation by endogenous adenosine and exogenous adenosine receptor agonists, and demonstrated that presence and absence of endogenous adenosine could play a key role in the adenosine A_1 receptor agonist-mediated ischemic cardioprotection in cardiac cell ischemia model. Synergistic cooperative cardioprotective interaction or "cross-talk" between adenosine $A_1$and $A_{2A/B}$ receptors during ischemic injury was demonstrated in a cardiac cell ischemia model. Role of endogenous adenosine in the synergistic activation of adenosine A_{2A}/A_{2B} receptors when the adenosine A_1 receptor is activated exogenously during reperfusion has been demonstrated using both pharmacological and targeted gene knockout interventions in the isolated mouse heart model. Work in the present thesis explored briefly the downstream cardioprotective signalling mechanism, which involved activation of extracellular signal-regulated kinase (ERK) 1/2 phosphorylation as a result of the synergistic activation of adenosine A_1 and A_{2A} receptor in a clinically relevant model of A_1 agonist intervention during reperfusion injury in the isolated mouse heart model. The major findings from the studies reported in this thesis are:

- Endogenous adenosine released during ischemic injury plays significant role in pharmacological preconditioning. Cardioprotective effects of adenosine A_1 agonists during ischemia are reduced in the absence of endogenous adenosine, whilst adenosine A_3 agonist-mediated ischemic protection was found similar in the presence or absence of endogenous adenosine in a cardiac cell ischemia model.
- Synergistic cardioprotection induced by adenosine A_1 receptor agonist involves cooperative activation of adenosine A_{2A} receptor by endogenous adenosine in a

cardiac cell ischemia model and in the isolated mouse heart ischemia reperfusion injury model.

- The synergistic "cooperative cardioprotection by adenosine A_1 and A_2 receptor" agonism perhaps relates to a more than additive effect of adenosine A_1 and A_2 receptor activation on downstream ERK1/2 phosphorylation signalling in isolated mouse heart model.
- A novel, highly selective adenosine A_1 partial agonist VCP28 is cardioprotective when administered during ischemia (pharmacological preconditioning) in a cardiac cell ischemia model and during reperfusion (pharmacological postconditioning) with minimal bradycardic effect compared to full adenosine A_1 agonist in the clinically relevant isolated rat heart model, which can mimic the *in-vivo* ischemia and reperfusion conditions. VCP28 is of interest as a potential therapy for cardiac ischemia reperfusion injury.
- A novel, highly selective adenosine A_3 receptor agonists VCP103, VCP439, VCP438, VCP486, VCP485 and VCP487 reduce ischemic cell death when administered during ischemia in a cardiac cell ischemia model. These novel compounds are of interest as a potential therapy for cardiac ischemia reperfusion injury.

6.2. Development and validation of nonlethal simulated ischemia model using H9c2(2-1) rat cardiac cell

In the present study, a nonlethal cardiac cell simulated ischemia model was developed and characterized for the screening of potential and novel, highly selective adenosine A_1 and A_3

receptor agonists as cardioprotective agents (described in Chapter 2). Various studies have reported cardiac cell simulated ischemia model for the evaluation of cardioprotection during ischemic condition (see Table 2.3). However, some of these studies have reported the use of lethal simulated ischemia buffer containing sodium cyanide, hydrogen peroxide to create ischemic environment in cardiac cells (Ohata *et al.,* 1994; Jung *et al.,* 2004) and longer exposure time of ischemic buffer from 16, 24, 48 to 72 hrs (Chapman *et al.,* 2002; Bonavita *et al.,* 2003; Tantini *et al.,* 2006). In the present study, HEPES based nonlethal simulated ischemia buffer has been utilized to develop a nonlethal ischemic condition in a cardiac cell. Ischemic damage in cardiac cells treated with selective adenosine A_1 and A_3 receptor agonists during ischemia was reversed as shown by the reduced cell death.

6.3. Cardioprotection by adenosine A_1 and A_3 receptor agonist when administered during ischemic injury

Ischemic preconditioning is the condition in which prior exposure to small episodes of nonlethal ischemia protects the heart from lethal ischemic episode. Similarly, infusion of pharmacological agents prior to lethal ischemic provides cardioprotection is known as pharmacological preconditioning. In the present study, we observed that pharmacological preconditioning with the selective adenosine A_1 and A_3 receptor agonist (CPA and IB-MECA, respectively; when administered during ischemic injury) reduced necrotic cell death in a cardiac cell ischemia model. We found that the novel and highly selective adenosine A_1 (Gregg *et al.*, 2007) and A_3 compounds (synthesized in the faculty) give cardioprotection when administered during ischemia (pharmacological preconditioning) in

our cardiac cell ischemia model (described in Chapter 2). These novel adenosine A_1 and A_3 compounds are of interest as a potential therapy for ischemia reperfusion injury.

6.4. Involvement of endogenous adenosine during ischemic cardioprotection

Endogenous adenosine released during ischemia plays a key role in cardioprotection-mediated by ischemic preconditioning mechanism. Reports suggest that exogenous agonist or endogenous adenosine-mediated activation of adenosine A_1 receptor during ischemia reduces cell death in a cardiac cell ischemia model (Stambaugh *et al.,* 1997; Safran *et al.,* 2001) and give cardioprotection by reduced infarct size and improved postischemic functional recovery in isolated mouse (Matherne *et al.,* 1997; Peart *et al.,* 2000; Flood *et al.,* 2002), rat (Lasley *et al.,* 1990; Headrick 1996), rabbit (Headrick 1996), and *in-vivo* rabbit hearts (Thornton *et al.*, 1992). Evidence shows that endogenous adenosine has minimal effect on adenosine A_3 receptors (Cohen *et al.*, 2008), since adenosine A_3 antagonists has shown no effect on ischemic cardioprotection-mediated by the endogenous adenosine (Headrick *et al.*, 2005). In the present study, we found that removal of endogenous adenosine from the cell culture environment by addition of adenosine deaminase (ADA; converts adenosine into inosine) increased the number of nonviable cells in cardiac cell ischemia model. Adenosine A_1 agonist (CPA) produced cardioprotection (reduced number of nonviable cells) in the presence of endogenous adenosine. However, removal of endogenous adenosine decreased the CPA-mediated cardioprotection in cardiac cell ischemia model. Interestingly, we observed that removal of endogenous adenosine had no effect in the cardioprotection-mediated by the adenosine A_3 agonist (IB-MECA) in cardiac cell ischemia model. This data suggests that endogenous adenosine give ischemic

preconditioning cardioprotection by activation of adenosine A_2 receptors in our cardiac cell ischemia model.

Studies show that hypoxic challenge increases the intracellular and extracellular inosine level in HL-1 cardiomyocyte (Naydenova *et al.*, 2008), and interstitial inosine increases significantly in isolated rat and rabbit hearts (Headrick 1996). Reports suggest that inosine released during ischemic condition is involved in the maintenance of homeostasis by increasing hepatic glucose level by activation of adenosine A_3 receptor (Guinzberg *et al.*, 2006). Inosine reduced ischemic brain injury in rats, and the protective effect of inosine was abolished in the presence of adenosine A_3 antagonist MRS1191, suggesting that inosine give protection against ischemia reperfusion injury by activation of the adenosine A_3 receptor (Shen *et al.*, 2005). Evidence shows that during ischemic conditions inosine stimulates mast cell degranulation by activation of adenosine A_3 receptor in guinea pig lung (Jin *et al.*, 1997). In the present study, we observed that adenosine A_3 agonist (IB-MECA)-mediated ischemic cardioprotection was not reduced compared to A_1 agonist (CPA), when endogenous adenosine was removed from the cell culture environment, possibly due to the inosine produced from the breakdown of endogenous adenosine activates adenosine A_3 receptor and maintained the level of ischemic cardioprotection in our cardiac cell ischemia model. There may be a possibility that inosine produced due to breakdown of endogenous adenosine maintains the ischemic cardioprotection by activation of adenosine A_3 receptor in our cardiac cell ischemia model.

6.5. Synergistic cooperative interaction (cross-talk) between adenosine A_1 and A_2 receptor during ischemia in cardiac cell ischemia model and during reperfusion in the isolated mouse heart model

In chapter 3, we observed that cardioprotection induced by adenosine A_1 receptor agonist during ischemia involves cooperative activation of adenosine A_{2A} and A_{2B} receptors by endogenous adenosine in a cardiac cell ischemia model (Urmaliya *et al.*, 2009). Adenosine A_1 agonist (CPA)-mediated cardioprotective effect was abolished by the selective adenosine A_{2A} and A_{2B} antagonists (ZM241385 and MRS1754, respectively), but not by the adenosine A_3 antagonist (MRS1191) in the presence of endogenous adenosine in a cardiac cell ischemia model. However, in the absence of endogenous adenosine ZM241385 and MRS1754 failed to block the CPA-mediated cardioprotection, suggest that endogenous adenosine-mediated activation of adenosine A_{2A} and A_{2B} receptor contributes to the cardioprotective effect of CPA during ischemic injury in our cardiac cell ischemia model. In chapter 3, we observed that adenosine A_3 agonist (IB-MECA)-mediated cardioprotection was not abolished by adenosine A_1 or A_{2A} or A_{2B} antagonists in cardiac cell ischemia model. We found that the combination of either adenosine A_{2A} (CGS21680) or A_{2B} (LUF5834) receptor agonists with adenosine A_1 receptor agonist (CPA) in the absence of endogenous adenosine increased ischemic protection as compared to CPA alone (described in Chapter 3). We further investigated whether this cooperative effect was seen in isolated mouse hearts with ischemia-reperfusion damage when CPA was given at reperfusion.

In chapter 4, we have found that adenosine A_1 receptor agonist (CPA) provides cardioprotection during reperfusion injury as indicated by increased postischemic cardiac

functional recovery, reduced infarct size and decreased LDH efflux, the effect was diminished not only by the adenosine A_1 antagonist DPCPX, but also by adenosine A_{2A} and A_{2B} antagonist (ZM241385 and MRS1754, respectively) in the wild type (WT) isolated mouse heart model. We observed that adenosine A_1 agonist (CPA) failed to give cardioprotection in the adenosine A_{2A} knockout (A_{2A}KO) isolated mouse heart model. The data in chapter 4 suggest that during reperfusion synergistic cooperative cardioprotection occurs when the adenosine A_1 receptor is activated by CPA and simultaneously adenosine A_{2A}/A_{2B} receptors were activated, presumably by endogenous adenosine, in the isolated mouse heart model. These data strengthen the existing evidence for A_1 / A_2 cooperative cardioprotection from Lasley and colleagues in *in-vivo* rat hearts (Lasley *et al.*, 2007) as well as our recent finding in a cardiac cell ischemia model (Urmaliya *et al.*, 2009), that adenosine A_1 receptor-mediated cardioprotection requires synergistic activation of adenosine A_{2A} and A_{2B} receptors by endogenous adenosine. Cooperative activation of adenosine A_1 and A_{2A} receptor appear to be required for maximal postischemic cardioprotection during ischemia and reperfusion injury using both pharmacological intervention and targeted gene knockout intervention in isolated mouse heart model (described in Chapter 4).

6.6. Development of ischemia reperfusion injury model in isolated mouse and rat hearts

To study the cardioprotection during ischemia reperfusion injury in isolated mouse and rat heart, ischemia reperfusion injury model has been developed in our laboratory. Based on empirical studies performed in our laboratory, we selected 30 min global ischemia followed

by 60 min reperfusion duration in isolated mouse and rat heart model (data not shown). The duration of ischemic condition selected was sufficient to develop reversible ischemic contracture in isolated hearts, which was reversed with the treatment by selective adenosine A_1 receptor agonist during reperfusion injury. Vehicle treated control mouse and rat isolated hearts shows reduced left ventricular developed pressure, increased EDP (contracture), reduced contractility (dP/dt_{max}) during reperfusion, the cardiac postischemic functions were recovered in the presence of selective adenosine A_1 agonist (CPA).

6.7. Cardioprotection induced by synergistic cooperative interaction between adenosine A_1 and A_2 receptor involves downstream activation of ERK1/2 phosphorylation signalling mechanism in mouse isolated heart model

Evidence suggests that adenosine receptors induce the downstream activation of ERK1/2 phosphorylation, which plays a key role in the adenosine receptor-mediated cardioprotection. Reports suggest that activation of adenosine A_1, A_{2A}, A_{2B} and A_3 receptor increases the ERK1/2 phosphorylation in isolated rat cardiomyocytes (Germack *et al.*, 2004), in CHO cells expressing human adenosine receptor subtypes (Schulte *et al.*, 2000), in isolated mouse hearts (Morrison *et al.*, 2007) and in *in-vivo* rat hearts (Reid *et al.*, 2005). In Chapter 4 we found that treatment with the selective adenosine A_1 agonist (CPA) improved cardioprotection concomitant with a significant increase in the downstream ERK1/2 phosphorylation signalling in WT isolated mouse hearts. However, CPA treatment did not have any effect on ERK1/2 phosphorylation signalling in A_{2A}KO isolated mouse hearts. We observed that CPA induced increased ERK1/2 phosphorylation signalling was significantly reversed by the adenosine A_{2A} antagonist ZM241385, but not by the adenosine

A_{2B} antagonist MRS1754 in WT isolated mouse hearts. In the present study we found that CPA treatment during reperfusion has shown no effect in ERK1/2 phosphorylation in A_{2A}KO isolated mouse hearts. The data suggest that for full ERK1/2 signalling though the activation of adenosine A_1 receptor, endogenous activation of adenosine A_{2A} receptor is required. Adenosine A_{2A} agonist (CGS21680) alone or in combination with CPA has shown no effect on ERK1/2 phosphorylation signalling, suggest that adenosine A_{2A} synergistic activation is maximally exerted by the endogenous adenosine released during ischemic condition in isolated mouse heart model (Figure 6.1; described in Chapter 4). In support of our findings, a study shows that mixed adenosine A_1/A_2 receptor agonist AMP579 offers cardioprotection by activation of ERK1/2 phosphorylation signalling, suggest the synergistic activation of adenosine A_1 and A_2 receptor involve in the downstream activation of ERK1/2 phosphorylation signalling (Kis *et al.*, 2003). Of course, these beneficial effects of AMP579 could be additive A_1 and A_2 effects.

The role of adenosine in PostC-mediated cardioprotection has been reported (Vinten-Johansen *et al.*, 2005). PostC cardioprotection involves various mechanisms include activation of reperfusion injury salvage kinase (RISK pathways; PI3K-Akt, ERK1/2, PKC, PGG) (Hausenloy *et al.*, 2005), activation of adenosine receptors, inhibition of mPTP opening (Hausenloy *et al.,* 2007; Hausenloy *et al.,* 2008), decreased mitochondrial calcium deposition, antioxidant mechanism, antiapoptotic mechanism, activation of mitochondrial K_{ATP} (mitoK_{ATP}) channels (Headrick *et al.,* 2000; Peart *et al.,* 2007; Kuno *et al.,* 2008), and attenuation of inflammatory response by inhibition of neutrophil and leukocyte adhesion to the endothelial cells (Hausenloy *et al.,* 2004; Vinten-Johansen *et al.,* 2005; Yellon *et al.,*

2005; Hausenloy *et al.,* 2006). Thus it is an extremely busy signalling environment, and difficult to dissect out individual signalling pathways involved in adenosine protection. The study in chapter 4 shows that cardioprotective ERK1/2 phosphorylation signalling was increased in the presence of CPA in WT isolated heart, but not in the A_{2A}KO hearts or in the presence of ZM241385, suggesting that adenosine A_1 receptor-mediated cardioprotection involves cooperative activation of adenosine A_{2A} and A_{2B} adenosine receptor by endogenous adenosine in our isolated mouse heart model.

6.7.1. Adenosine A_1 and A_2 receptor-mediated cardioprotection: synergistic interaction or additive effect?

In a cardiac cell ischemia model, adenosine A_1 receptor agonist (CPA)-mediated cardioprotection was abolished by adenosine A_{2A}, A_{2B} antagonist ZM241385 and MRS1754 respectively. We observed that CPA treatment improved post-ischemic cardiac functional recovery, reduced infarct size, decreased coronary LDH efflux and increased ERK1/2 phosphorylation in WT but not in A_{2A}KO isolated mouse heart ischemia reperfusion model. CPA-mediated cardioprotection was reversed by A_{2A}/A_{2B} antagonists in WT isolated mouse heart model. Threshold cardioprotection level was observed when adenosine A_1 receptor was activated alone or concomitantly with A_2 receptor by endogenous or exogenous agonists in a cardiac cell and isolated mouse heart model. In the absence of endogenous adenosine, combination of adenosine A_1 and $A_{2A/2B}$ receptor agonists provides significant cardioprotection in our cardiac cell ischemia model. However, in the absence of endogenous adenosine CPA-mediated cardioprotection was decreased below the threshold cardioprotection level in a cardiac cell ischemia model. Adenosine A_1

and A_2 activation together by exogenous agonist increased ERK1/2 phosphorylation in WT isolated mouse heart, which did not reach the statistical significance. Activation of adenosine A_2 receptor alone failed to give cardioprotection in our cardiac cell ischemia and isolated mouse heart model. The data presented in chapter 3 and 4 suggests that cardioprotection induced by adenosine A_1 receptors involves synergistic cooperative activation of adenosine A_2 receptors by endogenous adenosine in our cardiac cell ischemia and isolated mouse heart ischemia reperfusion models.

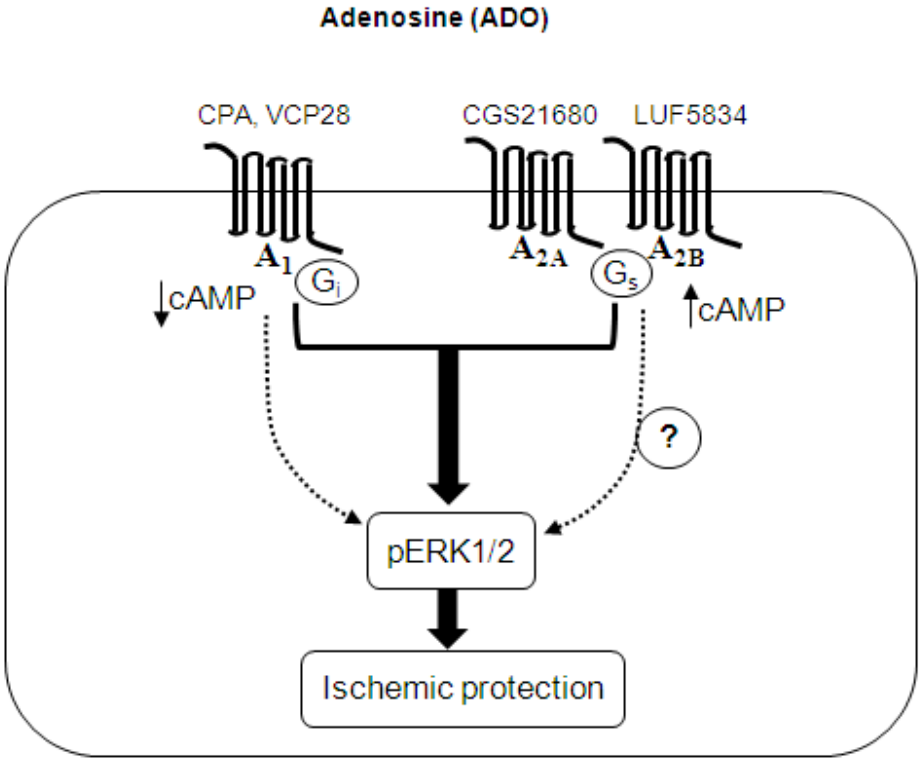


Figure 6.1. Cardioprotection induced by adenosine A_1 receptor activation involves cooperative activation of adenosine A_2 receptor by endogenous adenosine, which leads to ERK1/2 phosphorylation.

6.8. A novel, highly selective adenosine A_1 partial agonist VCP28 provide cardioprotection during both ischemia and reperfusion injury in a cardiac cell ischemia and isolated rat heart model

In the present study we have shown that the adenosine A_1 receptor partial agonist VCP28 decreases the number of nonviable cells when administered during ischemia in a cardiac cell ischemia model. VCP28 administration during reperfusion produced cardioprotection as seen by a significant reduction in infarct size with improvement in the postischemic cardiac function (increased contractility and left ventricular pressure) in isolated rat heart model. We observed a minimal effect of VCP28 on adenosine A_1 receptor-mediated heart rate reduction compared to the full adenosine A_1 agonist CPA in isolated rat heart (described in Chapter 5). Therefore VCP28 could be a better therapeutic agent for the cardioprotection during both ischemia and reperfusion injury.

VCP28 has high affinity for adenosine A_1 receptor (Ki value 50 nM for adenosine A_1 receptor) with 400 times more selectivity for adenosine A_1 receptor compared to adenosine A_3 receptor (Gregg *et al.*, 2007). VCP28 had approximately 100-fold lower potency for the adenosine A_1 receptor-mediated negative chronotropic effect, with approximately 1.5 times less efficacy compared to full adenosine A_1 agonist CPA in isolated rat heart (Chapter 5). Thus VCP28 has shown to be selective for the adenosine A_1 receptor, with minimal effect on heart rate reduction. Full A_1 agonists have been investigated for acute treatment of tachycardia and certain arrhythmias (Ellenbogen *et al.*, 2005), but the therapeutic window of these agents appears to be narrow. In the AMISTAD trial, two doses were used (50 and 70 μg/kg/min), the low dose having no effect on infarct size and the higher dose producing

modest reduction in infarct size. The authors of the AMISTAD II trial state that a low dose was used to "reduce the incidence of bradycardia and hypotension"(Ross *et al.*, 2005).

The partial A_1 agonist VCP28 have shown minimal effect in the adenosine A_1 receptor-mediated heart rate reduction effect compared to the full adenosine A_1 agonist CPA. Use of the adenosine A_1 agonist as a anti-arrhythmic agent is restricted because of the unwanted cardiovascular side effects such as bradycardia, AV block, atrial fibrillation (Elzein *et al.*, 2008). Reports have shown that use of the partial adenosine A_1 agonist CVT-2759 slows AV node conduction and heart rate without AV conduction block and bradycardic effect in isolated guinea pig heart (Wu *et al.*, 2001). In the present study data suggest that partial agonist VCP28 have shown cardioprotection comparable as full adenosine A_1 agonist without unwanted cardiovascular side effects in isolated rat hearts. VCP28 could possibly used as a lead molecule for the further investigation of its cardiovascular potential for therapeutic use during ischemia and reperfusion injury.

Cardioprotective effect of VCP28 shown by the reduced cardiac cell death and LDH efflux when administered during ischemia (Urmaliya *et al.*, 2009) was found to be similar in a cardiac cell ischemia model we reported earlier (Gregg *et al.*, 2007). The selective adenosine A_1 antagonist DPCPX abolished the VCP28-mediated cardioprotection in a cardiac cell ischemia model. In the absence of endogenous adenosine VCP28 mediated cardioprotection was decreased supported by our finding that A_1 agonists provide better cardioprotection in the presence of endogenous adenosine in which we observed increased cardioprotection of CPA in cardiac cell ischemia model (Urmaliya *et al.*, 2009).

Administration of CPA and VCP28 during reperfusion improved postischemic contractility (dP/dt_{max}) and left ventricular function, and reduced infarct size in isolated rat heart model. The cardioprotective effects of CPA and VCP28 were abolished in the presence of the adenosine A_1 receptor antagonist DPCPX. Various studies support that activation of adenosine A_1 receptor improve the postischemic cardiac function in isolated rat (Lasley *et al.,* 1992; Finegan *et al.,* 1996; Lozza *et al.,* 1997) and mouse hearts (Butcher *et al.*, 2007). However, in contrast studies have shown no cardioprotection by activation of adenosine A_1 receptor during reperfusion in isolated mouse heart (Peart *et al.*, 2002), *in-vivo* rat heart (Kin *et al.,* 2005a) and *in-vivo* rabbit heart (Baxter *et al.*, 2000). Despite the discrepancy in the adenosine A_1-mediated cardioprotection during reperfusion, possibly due to variation in the species and dose administered, our data suggest that pharmacological postconditioning with adenosine A_1 receptor agonist protects the heart from ischemia reperfusion injury in isolated rat heart model.

6.9. Study limitations, assessments and future directions

In the present study, endogenously released adenosine level was not quantified in cardiac cell ischemia model and in the isolated mouse heart model. Thus, it is not clear what concentration of endogenous adenosine is present at different times during and after ischemia, in addition to any exogenous agonist. Endogenous adenosine levels increase during various pathophysiological conditions such as hypoxia, ischemia and reperfusion injury (Ely *et al.,* 1992a; Headrick 1996; Headrick *et al.,* 2001b; Peart *et al.,* 2001; Talukder *et al.,* 2003). Reports suggest that endogenous adenosine plays role in adenosine A_1 receptor-mediated preconditioning cardioprotection in isolated mouse hearts (Matherne

et al., 1997; Peart *et al.,* 2000; Flood *et al.,* 2002), rat hearts (Lasley *et al.*, 1990) and in-vivo rat hearts (Headrick 1996). However, this limitation creates a scope for the future experimental lead to compare the effect of endogenous adenosine with the exogenous adenosine on adenosine A_2 receptor in a cardiac cell ischemia and isolated mouse heart model.

Genetically modified mice have been of great interest in the field of adenosine research presently to reveal the mechanisms involve in the cardioprotection and to explore the role of specific adenosine receptor subtypes responsible for the cardioprotection during ischemia and reperfusion injury. In the present study we used pharmacological as well as gene knockout mouse approaches to explore the cardioprotective effect involve synergistic interaction between adenosine A_1 and A_{2A}/A_{2B} adenosine receptor during ischemia and reperfusion injury (Chapter 4). However, the lack of the A_{2A} gene may have some effect on other receptor systems in terms of targeted expression (Gauthier *et al.*, 1998), other physiological system effects such as β-adrenergic system, effect on signalling of other proteins and downstream cascades, need to be considered. Functional genomics and the effect of targeted deletion of adenosine A_{2A} receptor gene in the mouse, and effect on second messenger activation, are of significant interest.

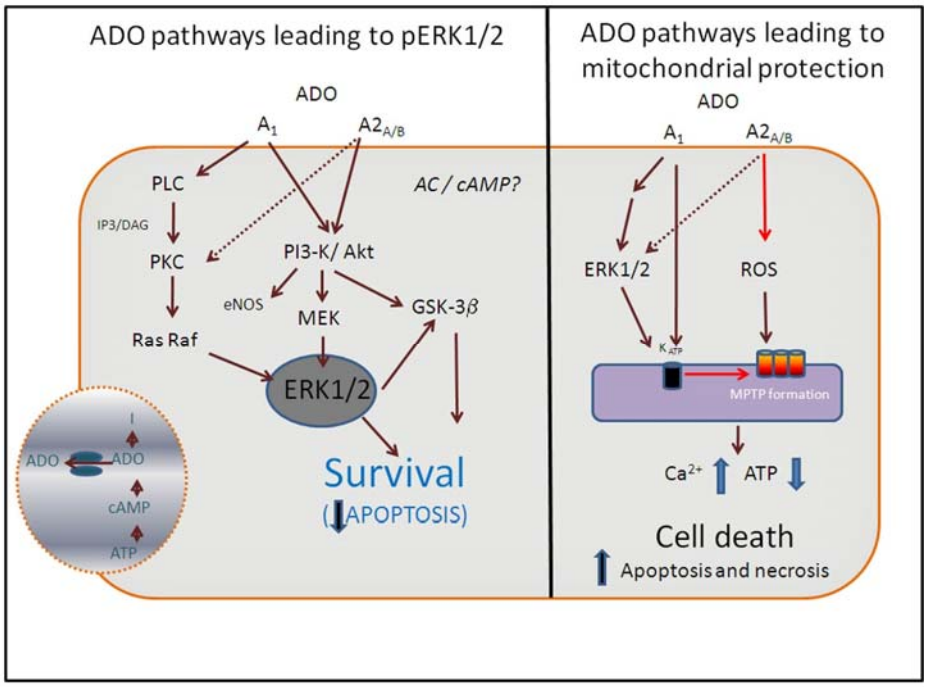


Figure 6.2. Proposed pathways to adenosine A_1 and A_2-mediated cardioprotection focusing on i) ERK1/2 phosphorylation and ii) mitochondrial effects. Red arrows indicate inhibitory effects.

The lack of knowledge of the signalling events between adenosine A_1 and A_2 receptor activation and ERK1/2 phosphorylation is perhaps the biggest limitations to understand the cardioprotection mechanism involved during ischemia and reperfusion injury. Figure 6.2 shows the proposed pathways of adenosine A_1 and A_2 receptor-mediated cardioprotection, which could further explore the cardioprotective mechanism involved during ischemia and reperfusion injury and the signalling pathways activated by the adenosine A_1 and A_2 receptors.

Work on human cardiomyocytes

Data from various well-controlled studies have shown both cardioprotection and a lack of cardioprotection from exogenous adenosine A_1 agonist at reperfusion. To avoid discrepancy in the findings of adenosine A_1 receptor-mediated cardioprotection from various animal species and to therefore focus on the clinical relevance of adenosine A_1 receptor activation at reperfusion, investigation of the nature of A_1 receptor-mediated protection in human cardiomyocytes are of significant interest for the translation of the present research outcome into the clinical setup.

In-vivo animal model to compare with a cardiac cell ischemia and isolated mouse heart model

In the present study, we demonstrated the cooperative cardioprotection by the activation of adenosine A_1 and A_{2A}/A_{2B} receptor in a cardiac cell and in isolated mouse heart model. However, comparison of the present finding in an *in-vivo* animal model using knockout mice would further reveal the nature of the synergy between adenosine A_1 and A_2 receptor during ischemia and reperfusion injury. The presence of humoral and cell mediated factors in an *in-vivo* animal model could possibly play important role during ischemia and reperfusion. In *in-vivo* animal study, echocardiography analysis can be used to explore the effect of adenosine receptor activation on the ventricular function along with measurement of cardiac functional parameters and ischemic markers released in the blood. Furthermore, it is of great interest to know that whether the interaction between adenosine A_1 and A_2 receptor involves receptor hetero or homodimerization and internalization of the adenosine receptor during ischemia and reperfusion injury. It would be of significant interest to know

the physical changes occur in the adenosine receptors during pathophysiological conditions such as ischemia and reperfusion injury.

Downstream second messenger signalling involve in the adenosine A_1 and A_2 receptor synergistic cardioprotection in in-vivo heart model

Adenosine receptor synergistic cardioprotection mechanism presumably involve activation of diverse second messenger downstream signalling pathways, such as activation of reperfusion injury salvage kinase (RISK) pathways including- ERK1/2, Akt, p38, PKC activation and mitochondrial protection such as inhibition of mPTP opening, and activation of mitochondrial K_{ATP} channel activation. The study of these downstream cardioprotective measures in an *in-vivo* model would be of significant interest to further extend the knowledge in the adenosine receptor-mediated cardioprotection.

Development of tissue specific adenosine receptor agonist

Adenosine A_1 receptor agonists have unwanted effects on cardiovascular function as well as in central nervous system. Moreover, nonspecific activation or overstimulation of adenosine receptor causes desensitization of the adenosine receptors (Elzein *et al.*, 2008). Development of tissue specific agonists, which activates adenosine receptor on specific tissue, would possibly minimize the unwanted side effect in cardiovascular and central nervous system.

6.10. Conclusions

In the present study, we demonstrate that (i) endogenous adenosine released during ischemic condition increases cardioprotection mediated by the exogenous activation of adenosine A_1 receptor in a cardiac cell ischemia model, furthermore that (ii) cardioprotection induced by adenosine A_1 receptor agonist involves cooperative activation of adenosine A_{2A} and A_{2B} receptors by endogenous adenosine in a cardiac cell ischemia and isolated mouse heart ischemia reperfusion models, (iii) synergistic "cooperative A_1 and A_2 ischemic protection" involves downstream activation of ERK1/2 phosphorylation signalling in isolated mouse hearts, (iv) novel and highly selective adenosine A_1 and A_3 receptor agonists (synthesized in the faculty) give cardioprotection when administered during ischemic condition, (v) a novel, highly selective adenosine A_1 receptor partial agonist VCP28 provide cardioprotection when administered during ischemia (pharmacological preconditioning) in a cardiac cell ischemia model and during reperfusion (pharmacological postconditioning) with greater efficacy and potency than pathways mediating acute cardiac effects, such as bradycardia, in the isolated rat heart model.

6.11. Clinical Perspective

"Adenosine is indeed the indicator of cell viability, the signal of life" (Engler 1991). Adenosine plays key role in the heart during normal physiological and, pathophysiological conditions such as ischemia and reperfusion injury (Berne 1963; Berne 1980; Ely *et al.,* 1992a). Despite the exploration by various research groups in many years, various aspects of the cardioprotective mechanisms of adenosine receptor subtypes are still unclear. Translation of the cardioprotection observed in the different animal models has been not

very successful into the clinical setting to reduce morbidity and mortality during ischemia and reperfusion injury.

Clinical observation shows that use of adenosine in the treatment of myocardial ischemia gives cardiovascular unwanted effects such as bradycardia and hypotension and overstimulation of adenosine receptor cause receptor desensitization. In the present thesis we have shown that a novel, highly selective partial adenosine A_1 agonist VCP28 administered during both ischemia and reperfusion is profoundly cardioprotective similar to a full adenosine A_1 agonist with minimal bradycardic effect in the isolated rat hearts. Our data from the present study suggest the clinical implication of a combined therapy of adenosine A_1 and A_2 receptor agonist and use of a partial adenosine A_1 agonist for the treatment of myocardial ischemia reperfusion injury in the clinical setting. Furthermore, to comprehend the potential of "cooperative A_1 and A_2 ischemic protection", our findings need a consideration to translate from research laboratory to the clinic, and furthermore, the potential of the synergistic interaction between adenosine A_1 and A_{2A}/A_{2B} receptor in cardioprotection during ischemia and reperfusion injury need to be investigated.

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