

# Modulation of the Akt/Ras/Raf/MEK/ERK pathway by A<sub>3</sub> adenosine receptor

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**Abstract** Downstream A<sub>3</sub> receptor signalling plays an important role in the regulation of cell death and proliferation. Therefore, it is important to determine the molecular pathways involved through A<sub>3</sub> receptor stimulation. The phosphatidylinositol-3-OH kinase (PI3K)/Akt and the Raf/mitogen-activated protein kinase (MAPK/ERK) kinase (MEK)/mitogen-activated protein kinase (MAPK) pathways have central roles in the regulation of cell survival and proliferation. The crosstalk between these two pathways has also been investigated. The focus of this review centres on downstream mediators of A<sub>3</sub> adenosine receptor signalling.

**Key words** adenosine · A<sub>3</sub> receptors · cell death · cell proliferation · cell signalling

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## Abbreviations

CHO-hA <sub>3</sub>	CHO cells transfected with the human recombinant A <sub>3</sub>
Cl-IB-MECA	<i>N</i> > <sup>6</sup> -(3-iodobenzyl)2-chloroadenosine-5'- <i>N</i> -methyluronamide
DPCPX	1,3-dipropyl-8-cyclopentylxanthine
ERK	extracellular signal-regulated kinase
GSK-3β	glycogen synthase kinase
IB-MECA	<i>N</i> > <sup>6</sup> -(3-iodobenzyl)adenosine-5'- <i>N</i> -methyluronamide
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase kinase
MRE 3008F20	5 <i>N</i> -(4-methoxyphenyl-carbomoyl)amino-8-propyl-2-(2-furyl)-pyrazolo-[4,3 <i>e</i> ]1,2,3-triazolo [1,5 <i>c</i> ] pyrimidine
MRE 2029F20	[ <i>N</i> -benzo[1,3]dioxol-5-yl-2-[5-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1 <i>H</i> -purin-8-yl)-1-methyl-1 <i>H</i> -pyrazol-3-yloxy]-acetamide]
NECA	5'- <i>N</i> -(ethyl)carboxamidoadenosine
pAkt	phosphorylated Akt
PI3K	phosphatidylinositol 3-kinase
PLC	phospholipase C
PKA	protein kinase A
SCH 58261	7-(2-phenylethyl)2-(2-furyl)pyrazolo[4,3 <i>e</i> ]1,2,3-triazolo[1,5 <i>c</i> ]pyrimidine
siRNA	small interfering RNA
siRNA <sub>A3</sub>	small interfering RNA that targets A <sub>3</sub> receptor mRNA

## Overview of the A<sub>3</sub> receptor signalling

A<sub>3</sub> adenosine receptors couple, via G proteins, to classical second-messenger pathways such as inhibition of adenylyl cyclase [1], stimulation of phospholipase C (PLC) [2] and

calcium mobilization [3]. The activation of these signals controls important biological events, such as cell survival [4]. In particular, the  $A_3$  receptor-mediated induction of apoptosis or protection from cell death has been demonstrated in various experimental models and systems [4]. Certainly,  $A_3$  receptors have an important role in the control of neuronal cell death [5, 6]. Neuroprotective activity has been demonstrated following chronic administration of the  $A_3$  receptor agonist *N*<sup>6</sup>-(3-iodobenzyl)adenosine-5'-*N*-methyluronamide (IB-MECA) to gerbils with cerebral ischaemia, thus supporting speculation that treatment with adenosine  $A_3$  receptor agonists may decrease the infarct size following focal brain ischaemia [7].  $A_3$  receptors have also been involved in cardioprotection during prolonged stimulated ischaemia by rescuing injured myocytes [8]. Activation of  $A_3$  adenosine receptor protects against doxorubicin-induced cardiotoxicity and can attenuate myocyte injury during hypoxia [9–12]. Furthermore,  $A_3$  adenosine receptor activation triggers phosphorylation of protein kinase B and protects rat basophilic leukemia 2H3 mast cells from apoptosis [13]. In human melanoma cells,  $A_3$  adenosine receptor triggers a pro-survival signal, and the blockade of  $A_3$  receptors by selective antagonists induces deleterious consequences [14].

In contrast, high concentrations of the agonists for  $A_3$  receptor can induce apoptosis. Different studies have suggested the involvement of the  $A_3$  receptor in adenosine-induced apoptosis using *N*<sup>6</sup>-(3-iodobenzyl)2-chloro-adenosine-5'-*N*-methyluronamide (Cl-IB-MECA) in rat astrocytes [15] and human astrocytoma ADF cells [16], human peripheral blood mononuclear cells, both myeloid and lymphoid cells (U937, HL60) [17, 18] and cardiac myocytes [19, 20]. However, further investigations on CHO cells transfected with the human recombinant  $A_3$  adenosine receptors (CHO-h $A_3$ ) and on mast and lymphoma cell lines [13, 21–23] revealed no involvement of  $A_3$  receptors on induction of cell death, suggesting that the  $A_3$  receptor signalling is highly dependent on the cell-specific or tissue-specific context.

The molecular pathways sustaining the action of  $A_3$  receptor on cell survival have been defined. The present review will focus on the Akt-mediated signalling and the mitogen-activated protein (MAP) kinase cascades as principal pathways regulated by the  $A_3$  receptor.

### The $A_3$ receptor and the Ras-Raf-MEK-ERK signal transduction cascade

The  $A_3$  receptor signalling cascade has been demonstrated in different cellular models. Firstly, it has been suggested that  $A_3$  receptor modulates mitogenesis and the activation of ERK1/2 in human foetal astrocytes [24]. Physiological concentrations of adenosine (10–100 nM) have been

demonstrated to cause an increase in phosphorylation of ERK1/2 after 5 min in CHO cells transfected with any one of the four adenosine receptors [25]. Levels of adenosine reached during ischaemia (3  $\mu$ M) induce a more pronounced, but still transient, activation of ERK1/2. Thus, all the human adenosine receptors transfected into CHO cells are able to activate ERK1/2 at physiologically relevant concentrations of the endogenous agonist. Both 5'-*N*-(ethyl)carboxamido-adenosine (NECA) and the endogenous agonist (adenosine) lead to a time- and dose-dependent increase in ERK1/2 phosphorylation, at concentrations as low as 10 nM to 30 nM [25]. Moreover, these studies show that  $A_3$  receptor signalling in CHO-h $A_3$  cells leads to stimulation of ERK1/2 activity. In particular,  $A_3$  receptor signalling to ERK1/2 depends on  $\beta\gamma$  release from PTX-sensitive G proteins, PI3K, Ras and MEK [26]. Functional  $A_3$  receptors activating ERK1/2 have also been described in microglia cells [27]. In this cell model, by selectively stimulating the  $A_3$  receptor in both primary mouse microglia cells and in the N13 microglia cell line with the agonist Cl-IB-MECA, it has been found to be a biphasic, partly Gi protein-dependent influence on the phosphorylation of the ERK1/2.

However, different results have been obtained on this issue. In the human melanoma A375 cell line it has been demonstrated that Cl-IB-MECA was unable to activate ERK phosphorylation, while the  $A_3$  antagonists are able to improve the activity of MEKs [14]. Similar results were obtained in murine melanoma cells [28]. Furthermore, recently, we have found that  $A_3$  adenosine receptor stimulation inhibits cell proliferation by impairment of ERK kinase activation [29]. We studied the changes in ERK1/2 phosphorylation after treatment of melanoma cells with Cl-IB-MECA 10  $\mu$ M in a time-course experiment. Interestingly, prolonged inhibition of ERK1/2 phosphorylation was induced after the treatment of melanoma cells with Cl-IB-MECA, and this effect was sustained for 2 h after Cl-IB-MECA treatment. The kinetics of ERK1/2 phosphorylation reduction was very rapid: Cl-IB-MECA appeared to impair ERK1/2 phosphorylation after 5 min. Furthermore, we investigated the dose-dependence of Cl-IB-MECA to inhibit ERK1/2 phosphorylation. We found that the inhibition of ERK1/2 phosphorylation was produced by Cl-IB-MECA with an EC<sub>50</sub> of  $4.3 \pm 0.5 \mu$ M and  $2.2 \pm 0.3 \mu$ M for ERK-2 and ERK-1, respectively. The effect of Cl-IB-MECA was mediated by  $A_3$  receptor because, while the  $A_1$ ,  $A_{2A}$  and  $A_{2B}$  receptor antagonists were not able to prevent ERK1/2 inhibition induced by Cl-IB-MECA, the selective  $A_3$  receptor antagonist MRE 3008F20 (0.1  $\mu$ M) abrogated the Cl-IB-MECA-induced inhibition of ERK1/2 activation. Furthermore, we found that the inhibition of  $A_3$  receptor expression is sufficient to block Cl-IB-MECA-induced inhibition of ERK1/2 phosphorylation levels. These results emphasize the role of  $A_3$  receptors as

inhibitors of ERK activation and clearly show the connection between A<sub>3</sub> receptor stimulation and MEK/MAPK signalling in melanoma cells.

To explain why different results have been obtained on this issue it could be suggested that this mechanism may be peculiar for melanoma cells, having a misregulation of proliferative pathways, as A<sub>3</sub> receptors increased ERK1/2 phosphorylation in CHO-hA<sub>3</sub> cells in a dose-dependent manner [26] and induced a biphasic effect on the phosphorylation levels of ERK1/2 on microglia cells [27]. Further studies in other different cell systems will enhance our understanding of the role of A<sub>3</sub> receptors in the modulation of mitogenic signalling.

Furthermore, it has been shown that MAPK activation is involved in A<sub>3</sub> receptor regulation, both contributing to direct phosphorylation and controlling G protein-coupled receptor (GPCR) kinase protein membrane translocation, which are involved in receptor phosphorylation. Thus, an active MAPK pathway appears to be essential for A<sub>3</sub> receptor phosphorylation, desensitization and internalization [30].

### The A<sub>3</sub> receptor and the Akt signal transduction cascade

Another important pathway that is triggered by adenosine via A<sub>3</sub> receptor is the PI3K/Akt pathway [4]. One of the crucial downstream targets of PI3K is the serine/threonine kinase Akt. Active Akt causes a variety of biological effects, including suppression of apoptosis by phosphorylation and inactivation of several targets along pro-apoptotic pathways [31]. In particular, activated Akt is able to phosphorylate a variety of downstream substrates, e.g., the pro-apoptotic molecule Bad, caspase-9, the forkhead family transcription factors, I-K (a kinase that regulates the NF-κB transcription factor) and Raf.

Recent studies have demonstrated that protein kinase A (PKA) and PKB/Akt phosphorylate and inactivate glycogen synthase kinase 3β (GSK-3β), a serine/threonine kinase acting as a key element in the Wnt signalling pathway. In its active form, GSK-3β suppresses mammalian cell proliferation [28]. Only one study has indicated that the A<sub>3</sub> agonist IB-MECA is able to decrease the levels of PKA, a downstream effector of cAMP, and of the phosphorylated form of PKB/Akt in melanoma cells. This implies the deregulation of the Wnt signalling pathway, generally active during embryogenesis and tumorigenesis to increase cell cycle progression and cell proliferation [28]. In contrast, several studies have indicated the ability of A<sub>3</sub> receptor to activate Akt [4]. There is evidence that A<sub>3</sub> adenosine receptor activation triggers phosphorylation of PKB/Akt, protecting rat basophilic leukemia 2H3 mast cells from apoptosis by a pathway involving the βγ subunits of G<sub>i</sub> and PI3K-β. This process is blocked by pertussis toxin

and wortmannin [13]. More recently, it has been demonstrated that A<sub>3</sub> receptors trigger increases in Akt phosphorylation in rat cardiomyocytes via a G<sub>i</sub>/G<sub>o</sub>-protein and tyrosine kinase-dependent pathway [32]. An elegant study has recently documented a role of adenosine A<sub>3</sub> receptor in cell survival signalling in resveratrol preconditioning of the heart. This study provides evidence that resveratrol preconditions the heart through the activation of adenosine A<sub>1</sub> and A<sub>3</sub> receptors, transmitting a survival signal through the PI3-kinase-Akt-Bcl2 signalling pathway [33]. Further studies indicate that A<sub>3</sub> receptor activation can decrease IL-12 production, which is also mediated by interfering with PKB/Akt [34, 35]. Using human melanoma A375 cells we showed that A<sub>3</sub> adenosine receptor stimulation results in PI3K-dependent phosphorylation of Akt. We found that serum-deprived A375 melanoma cells had no basal Akt phosphorylation, whereas the A<sub>3</sub> receptor agonist Cl-IB-MECA treatment resulted in the phosphorylation of Akt at the Ser 573 phosphorylation site. In particular, we have demonstrated that the antiproliferative effect of Cl-IB-MECA is mediated by a PLC-PI3K-Akt signalling pathway [29].

### Crosstalk between MAPK and PI3K/Akt signalling pathways, and modulation by the A<sub>3</sub> receptor

Crosstalk between the PI3K and the Raf/MEK/ERK pathways has been reported on multiple levels, with some research stating that PI3K activity is essential for induction of Raf/MEK/ERK activity [31, 36]. Additional studies suggest that the PI3K pathway enhances and/or synergizes with Raf/MEK/ERK signalling to provide a more robust signal through the lower components of the MAPK cascade (i.e., ERK). However, there is conflicting evidence that states that Akt is able to phosphorylate Raf, thereby efficiently abrogating Raf activity on downstream substrates [37–41].

Considering the pleiotropic substrates and the ubiquitous expression of ERK, cell specific regulation must occur to ensure conduction of the appropriate signal.

Recently, we investigated the role of the adenosinergic system in the regulation of ERK activation in melanoma cells. We focused our attention on melanoma cells because the importance has been demonstrated of small autacoids, primarily thought to be neurotransmitters, in mediating a variety of biological activities in the skin [14, 42–45]. In particular, several studies have indicated that adenosine, via stimulation of its receptors, is involved in cell proliferation and cell death. In agreement with these studies, we also found that A<sub>3</sub> receptor inhibits human melanoma A375 cell line proliferation [42].

The activities of Akt or MAPK, or both, are elevated in many cancer cells and are known to play critically important roles in cellular proliferation [31, 36, 46]. On

the basis of this consideration, we tested the hypothesis that  $A_3$  receptor stimulation regulates cell growth signalling via the ERK1/2 and/or the Akt pathway in melanoma cells.

We found that Akt phosphorylation mediated by CI-IB-MECA induced Raf phosphorylation at an inhibitory phosphorylation site on Ser 259 of Raf. As a consequence, CI-IB-MECA inactivated Raf in a dose- and time-dependent manner. Interestingly, CI-IB-MECA stimulation resulted in a time- and dose-dependent reduction in ERK1/2 phosphorylation.

We examined whether any crosstalk existed between ERK1/2 and Akt pathways in A375 melanoma cells. The classical MAPK cascade leads from the Ras kinases to the MAPKK MEK1/2. There is evidence that Akt is able to phosphorylate Raf, thereby efficiently abrogating Raf activity on downstream substrates [39, 41, 47]. We studied the effects of  $A_3$  receptor stimulation on the proliferation of melanoma cells in the presence of specific inhibitors of the PI3K and Akt signal transduction pathways. We could effectively block the CI-IB-MECA-induced reduction of ERK1/2 phosphorylation with an inhibitor of PI3K. Indeed, application of CI-IB-MECA in combination with PI3K inhibition resulted in a clear increase of ERK1/2 phosphorylation when compared with P-ERK1/2 in the presence of CI-IB-MECA alone. These data suggest that the Ras-Raf-MEK-ERK pathway is normally activated by  $A_3$  receptor stimulation, as is the PI3K-Akt route. It is clear that these apparently separate routes should actually interact.

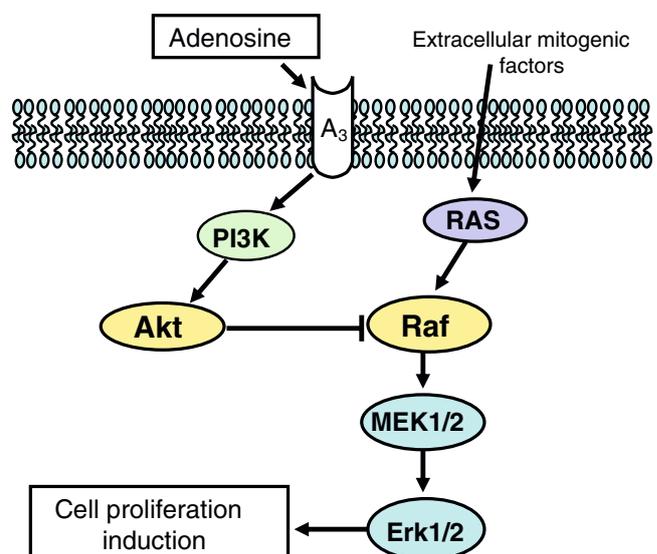
In order to investigate the functionality of  $A_3$  receptors expressed in melanoma cells we used the selective adenosine analogue CI-IB-MECA. It is not clear whether the growth inhibitory action of micromolar concentrations of the  $A_3$  receptor agonist CI-IB-MECA is due to its role as an extracellular ligand for cell surface receptors or whether it acts intracellularly as a second messenger. In addition, this agonist may, in high concentrations, activate  $A_1$  receptors, which, however, when compared with  $A_3$  receptors, are expressed at low level in A375 cells ( $B_{MAX} = 23 \pm 7$  fmol  $mg^{-1}$  of protein and  $B_{MAX} = 291 \pm 50$  fmol  $mg^{-1}$  of protein for  $A_1$  and  $A_3$ , respectively) [48]. Thus, the effects we report here on melanoma cell proliferation and on ERK1/2 phosphorylation induced by CI-IB-MECA using high ( $10^{-6}$  M) concentrations of CI-IB-MECA are almost certainly due to  $A_3$  receptor stimulation. In particular, the effects of CI-IB-MECA on cell proliferation and on ERK1/2 phosphorylation are not mediated by  $A_1$ ,  $A_{2A}$  or  $A_{2B}$  receptors. In support of this conclusion, DPCPX, SCH 58261 and MRE 2029F20, adenosine receptor antagonists highly selective for  $A_1$ ,  $A_{2A}$  and  $A_{2B}$  receptors [49, 50], respectively, did not block the inhibitory effect of  $A_3$  receptor stimulation on cell proliferation and on P-ERK1/2 modulation. On the contrary, the effects on cell proliferation and on P-ERK1/2 modulation were inhibited

by the  $A_3$  receptor antagonist, MRE 3008F20. Furthermore, the CI-IB-MECA-induced effects on cell proliferation and ERK1/2 phosphorylation in human A375 melanoma cells were abolished in cells in which  $A_3$  receptor protein was knocked down by si-RNA $_{A_3}$  treatment, when compared with wild-type cells. These findings, together with the specificity of the agonist used, makes us confident that the effects are due to the  $A_3$  receptor subtype.

$A_3$  receptor stimulation inhibits the proliferation of melanoma cells partly by a PLC-sensitive mechanism. Pretreatment of cells with a PLC- $\gamma$  inhibitor strongly abrogated the CI-IB-MECA effect on cell proliferation and on ERK1/2 phosphorylation, suggesting a critical role for PLC- $\gamma$  in  $A_3$  receptor signalling. Furthermore, pretreatment of A375 cells with a PI3K inhibitor and an Akt inhibitor impaired CI-IB-MECA-induced inhibition of cell proliferation and the effects of  $A_3$  receptor stimulation on Raf, MEK1/2 and ERK1/2 phosphorylation. These data suggest that the  $A_3$  adenosine receptor signals through a pathway that includes PI3K-Akt. In contrast, Ras was not activated. These results confirm that, in A375 cells,  $A_3$  receptors decrease MEK1/2-ERK1/2 phosphorylation and cell proliferation via the inhibition of Raf by a PI3K-Akt pathway, without affecting Ras.

Our results indicate that CI-IB-MECA acts extracellularly as a first messenger for cell surface receptors. In A375 cells the inhibition of PLC-PI3K-Akt signalling is able to block the effect of  $A_3$  receptor stimulation on cell proliferation, suggesting that, in the melanoma cell system, an inhibitory connection between PLC-PI3K-Akt and ERK1/2 is present (Figure 1).

Finally, we have described the molecular mechanism sustained by CI-IB-MECA interfering with cell prolifera-



**Figure 1**  $A_3$  adenosine receptor stimulates PI3K-dependent phosphorylation of Akt, leading to the reduction of basal levels of ERK1/2 phosphorylation that, in turn, inhibits cell proliferation

tion. CI-IB-MECA, via A<sub>3</sub> adenosine receptor binding, activates PLC-PI3K-Akt signalling that, in turn, reduces P-ERK1/2 levels necessary for cell proliferation. As a consequence, cells accumulate in G<sub>0</sub>/G<sub>1</sub> cell cycle phases, and low level of DNA incorporation is observed.

Further definition of the pathways leading to ERK1/2 inactivation, and translation into an *in vivo* model, are required to clarify whether adenosine signalling *in vivo* has characteristics similar to those observed in this *in vitro* model. In this scenario the regulation of ERK1/2 by A<sub>3</sub> receptor and PI3K would represent an important aspect of adenosine signalling.

Although it is possible that the signalling pathways may be different in other cellular backgrounds, it seems likely that the biological events regulated by adenosine A<sub>3</sub> receptors under physiological and pathophysiological conditions may depend not only on changes in cAMP and Ca<sup>2+</sup> but also on mitogenic signalling via ERK1/2 and Akt.

## Conclusions

Work by several groups, including ours, has shown a modulation of the Akt/Ras/Raf/MEK/ERK signalling pathway by adenosine. We propose that this modulation is essential to determine the final physiological response of A<sub>3</sub> adenosine receptor activation.

Of great interest are the different effects of adenosine on the Akt/Ras/Raf/MEK/ERK signalling pathway modulation in different cells.

Interestingly, adenosine may be, on the one hand, preventing the proliferative activation of ERK1/2 by blocking Raf through Akt activation and, on the other hand, stimulating PI3K activation in order to induce cell survival.

We believe that the opposite effects of adenosine are due to the presence of more than one receptor subtype in the same cellular system, each mediating seemingly opposite actions, e.g., having conflicting actions on cell proliferation and cell death. As a consequence, the net effect may not be defined. It is clear that adenosine concentration may determine the pattern of differential activation of co-expressed receptor subtypes. One adenosine receptor may also be coupled to more than one G protein. Several studies are helping to show if such coupling is physiologically important [51].

Further work to determine the various physiological responses mediated by A<sub>3</sub> receptor stimulation through the regulation of the Akt/Ras/Raf/MEK/ERK pathway will help us to understand the role played by adenosine in cell proliferation and cell death.

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