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Inhibition of mitochondrial permeability transition and release of cytochrome *c* by anti-apoptotic nucleoside analogues

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Abstract

We have investigated whether nucleoside drugs that induce or protect neurones against apoptosis might directly activate or inhibit mitochondrial permeability transition (mPT) since opening of the mPT pore can promote release of cytochrome *c* and apoptosis, while its closure can prevent these changes. We found that the pro-apoptotic pyrimidine analogues cytosine β-D-arabinofuranoside and cytosine β-D-arabinofuranoside 5′-triphosphate, which activated apoptosis in post-mitotic neurones without incorporation into nuclear DNA, induced rapid calcium-dependent mitochondrial swelling of isolated liver mitochondria in a dose-dependent manner. Induction of up to 50 and 80%, respectively, of maximal swelling induced by high calcium was obtained at 1 mM concentrations, which also promoted a 17-fold increase in the release of cytochrome *c*. Both activities were inhibited by cyclosporine A to unstimulated levels; dCTP had no effect. In contrast, the anti-apoptotic adenine analogues, 3-methyladenine (3-MA) and olomoucine (but not iso-olomoucine), inhibited swelling induced by calcium or phenylarsine oxide in a dose-dependent manner at concentrations that protect neurones from apoptosis. Both compounds also inhibited the release of cytochrome *c* (by 82%, 20 mM 3-MA and 95%, 0.9 mM olomoucine), similar to the inhibition obtained with cyclosporine A, and 5 mM ADP or ATP. Similar inhibitory effects with olomoucine and 3-MA were found in isolated heart mitochondria. These studies identify the mPT as an important target for hitherto untested pro- and anti-apoptotic nucleoside-based drugs and suggest that screening for mPT modulation is an important component in the validation of a drug's mechanism of action.

Keywords: Apoptosis; Autophagy; 3-Methyladenine; Cytosine arabinoside; Olomoucine; Mitochondria; Cytochrome *c* release

1. Introduction

Several chemotherapeutic agents that kill cells by apoptosis have been shown to act directly on the mitochondria by inducing mPT (reviewed in [1]), making mPT activation an attractive target for anticancer therapies. Amongst the nucleosides, the adenosine derivative dATP has been shown to promote release of cytochrome *c* from isolated mitochondria in a manner that is inhibited by the cyclophilin D inhibitor cyclosporine A (CsA), indicating mPT and possibly adenosine nucleotide transporter (ANT) involvement [2]. In addition, chloro and fluoro derivatives of deoxyadenosine metabolites were shown to reduce mitochondrial membrane potential in whole cells [3], suggesting possible involvement of mPT in addition to activating Apaf-1 *in lieu* of dATP [4]. Little is known about the efficacy of pyrimidines in mPT regulation, although agents such as cytosine β-D-arabinofuranoside (araC) that are used clinically are also capable of inducing apoptosis in post-mitotic neurones [5–7] without their being incorporated into nuclear DNA [5,6] and can be toxic to the nervous system [7].

In contrast to exploration of the pro-apoptotic effects of some nucleoside analogues, little work has been done to investigate the possibility that adenine-based analogues that prevent neuronal apoptosis mimic the actions of ADP/ATP, and inhibit mPT. This possibility is especially important as CsA treatment, which can be neuroprotective in rodent models of brain trauma [8], ischaemia [9] and

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Abbreviations: araC, cytosine β-D-arabinofuranoside; araCTP, cytosine β-D-arabinofuranoside 5′-triphosphate; CsA, cyclosporine A; mPT, mitochondrial permeability transition; 3-MA, 3-methyladenine.

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hypoglycaemia [10] may also promote neurotoxicity [11–14], and its delivery to the CNS may be problematic [15] due in part to its effects on the blood–brain barrier [16,17]. One candidate adenine analogue of special interest is 3-MA. 3-MA is best known as an inhibitor of autophagosome formation [18], but it has also been shown to induce alkalisation of lysozomes [19] and to inhibit various kinase activities [20]. 3-MA also inhibits apoptosis induced by TNFα in leukaemic cells [21] and by NGF deprivation or araC treatment in SCG neurones [20]. Little is known about the metabolic fate of 3-MA in the cells. It has been proposed that mPT pore opening is the trigger for activation of autophagy [22,23]. Since mPT pore activity can cause subsequent release of mitochondrial proteins such as cytochrome *c*, we hypothesised that 3-MA might be inhibiting both autophagy and apoptosis (and hence preserve mitochondrial function) by inhibiting mPT.

Olomoucine is another adenosine analogue which has high affinity as a cdk inhibitor [24]. At higher concentrations it also inhibits neuronal apoptosis [25,26]. Maas *et al.* [25] have suggested that the neuroprotection is mediated by inhibition of c-Jun N-terminal kinase (JNK) activity, but whether this activity is sufficient to account for the protection observed is not entirely clear.

We have, therefore, tested these two hypotheses—that araC and its derivatives induce mPT pore opening, and that 3-MA or olomoucine inhibit it—using isolated rat liver mitochondria. The effects of olomoucine and 3-MA have also been tested in heart mitochondria that respond to ischaemia by opening of the mPT [27]. To follow mPT pore activity, we have measured mitochondrial swelling and cytochrome *c* release. Here, we show that araC derivatives induce Ca^{2+}-dependent, CsA-inhibitable mPT and cytochrome *c* release, whereas 3-MA and olomoucine inhibit Ca^{2+}- and oxidant-induced mPT.

2. Materials and methods

2.1. Preparation of mitochondria

Liver mitochondria were prepared from male Wistar rats by homogenisation in medium containing 250 mM sucrose, 5 mM Tris–HCl, 5 mM EGTA (pH 7.4) and subsequent differential centrifugation as described previously [28]. The pellet was washed one time in EGTA-free sucrose/Tris medium and resuspended in the EGTA-free medium.

2.2. Assay for mPT

Mitochondria (1 mg protein) were incubated in 1.5 mL of incubation buffer in a stirred cuvette at 37° and the change in absorption at 540 nm was recorded continuously using a Unicam 8625 UV/VIS spectrophotometer. Light scattering by the mitochondria decreases as their volume increases during the swelling process [30]. The incubation buffer contained 120 mM KCl, 50 mM HEPES and 5 mM nitrilotriacetic acid (pH 7.0), with 1 mM pyruvate and 1 mM malate added as substrates for mitochondrial respiration. Threshold levels of swelling were determined by incubating mitochondria in the presence of 0.48 mM KH_2PO_4 and a series of small increments of 10 μM $CaCl_2$ until the mitochondria were sufficiently sensitised so that one further increment induced maximal swelling within 5–8 min. The mitochondria were examined periodically throughout the experiment by retesting the effects of the subthreshold concentrations of $CaCl_2$ determined at the beginning of the experiment (usually 30–40 μM) to ensure that no changes had taken place during the experiment. Heart mitochondria (0.1 mg) were incubated in 200 μL of buffer containing 110 mM KCl, 50 mM creatine, 5 mM KH_2PO_4, 10 mM Tris–HCl, 5 mM nitriloacetic acid, 2.24 mM $MgCl_2$, with 1 mM pyruvate and 1 mM malate (pH 7.2), and were induced to swell with 0.1 mM $CaCl_2$. To test the effects of mPT inducers and inhibitors, the drugs were added to the incubation medium just before or immediately after addition of mitochondria. AraC, cytosine β-D-arabinofuranoside 5′-triphosphate (araCTP), dCTP, 3-MA, ATP and ADP were obtained from Sigma UK. Olomoucine, iso-olomoucine and CsA were purchased from Calbiochem. Drugs were carefully buffered and tested to ensure they did not alter the pH of the incubation buffer before and after calcium was added. To quantify the relative amount of swelling, the difference between absorbance values before $CaCl_2$ addition and those induced after 10-min treatment with the drug were measured and the percentage of change was calculated relative to the change in absorbance measured after maximal swelling was induced by 100 μM $CaCl_2$ final concentration (considered as 100%).

2.3. Western blot for cytochrome c release

At the end of the swelling assay, the mitochondrial suspension was pelleted immediately at 10,000 *g* for 3 min and an aliquot of 10 μL (liver) or 20 μL (heart) was analysed by SDS/PAGE on 14% acrylamide gels. Proteins were transferred to a nitro-cellulose membrane and probed with mouse antibody to cytochrome *c* (Pharmingen 65981A) followed by ECL-based detection (liver) or an infra-red detection (heart) system (Li-Cor Biosciences) using an Alexa-680-conjugated secondary antibody (Molecular Probes). The intensity of bands was quantified by densitometry using a Leica Q500 image analysis system (liver) or using the Li-Cor software (heart).

2.4. Assay for mitochondrial respiration

Respiration was measured using a Clark-type oxygen electrode in the same medium as that used for the swelling experiments. Mitohcondrial state 3 respiration was achieved

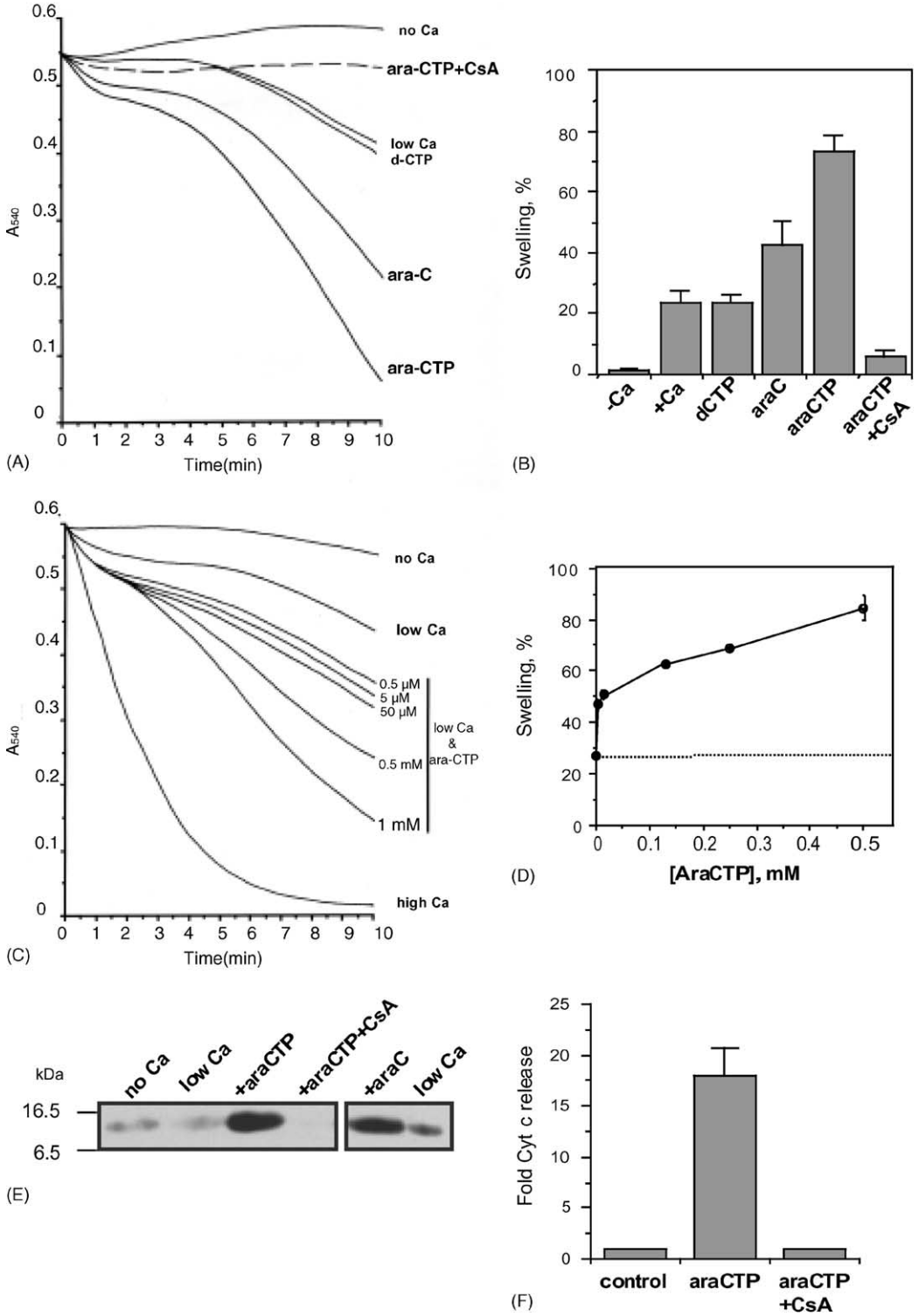
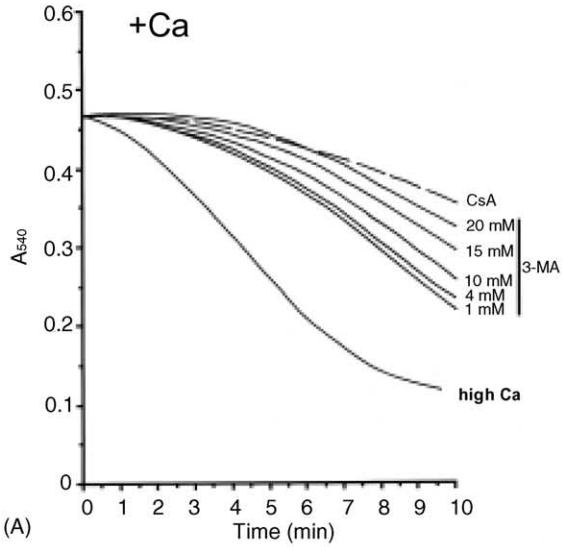
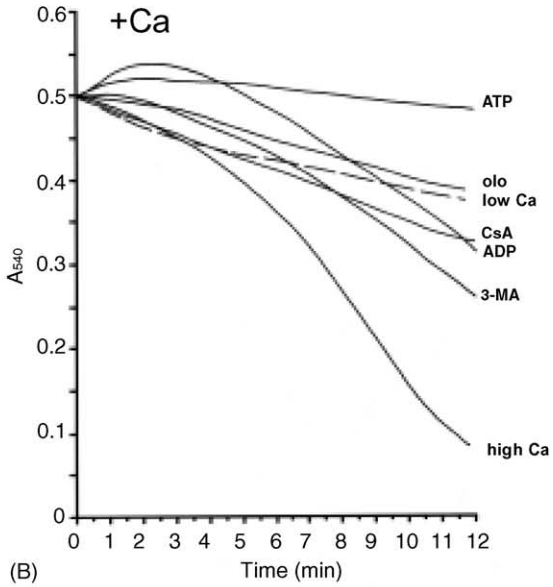


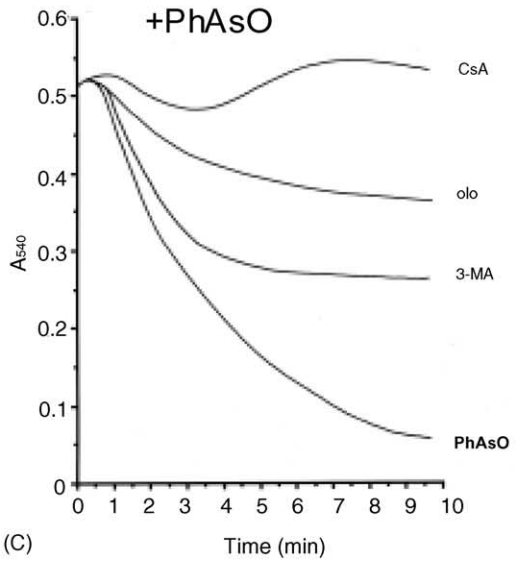
Fig. 1. AraC and araCTP, but not dCTP, activate mitochondrial swelling and promote the release of cytochrome *c*. (A) Mitochondria were pre-incubated without (“low Ca”) or with media containing 1 mM araC, araCTP, or dCTP, or with 3.2 μM CsA and 1 mM araCTP (dashed line) and then loaded with 0.48 mM KH_2PO_4 and 30 μM $CaCl_2$ at time zero; “no Ca” indicates untreated mitochondria. (B) The differences in absorbance readings between 0 and 10 min were used to calculate percentage swelling, 100% being the swelling induced with 50–100 μM $CaCl_2$. Results are mean ± SD from four independent experiments ($P < 0.02$, araC vs +Ca; $P < 0.001$, araCTP vs +Ca, two-tailed Student’s *t*-test). (C) Swelling induced by araCTP is dose dependent. Different concentrations of araCTP were added and the swelling process was followed as in (A); “high Ca”, swelling in the presence of 0.48 mM KH_2PO_4 and 100 μM $CaCl_2$. (D) Percent swelling was calculated as in (B) after addition of the indicated concentrations of araCTP. Results show mean ± range from two independent experiments. Dashed line shows swelling in the presence of low concentrations of $CaCl_2$. (E) At the end of the swelling assay, an aliquot from the clarified supernatants of the mitochondrial suspensions were analysed for cytochrome *c* content by western blotting. Concentrations and conditions for lanes labelled—no Ca, low Ca, AraC, araCTP and CsA—were as in panel (A). Equal input of mitochondria is indicated by the identical absorbance readings at time zero. (F) Densitometric reading of band intensities normalised to the average value obtained from the control sample (mean ± range of two independent experiments).


+Ca
A540
Time (min)
CsA
20 mM
15 mM
10 mM
4 mM
1 mM
3-MA
high Ca

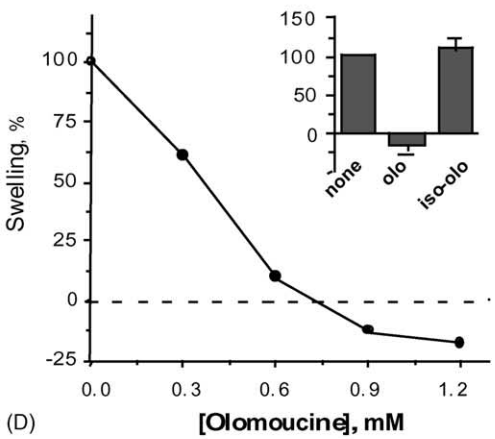
(A)


+Ca
A540
Time (min)
ATP
olo
low Ca
CsA
ADP
3-MA
high Ca

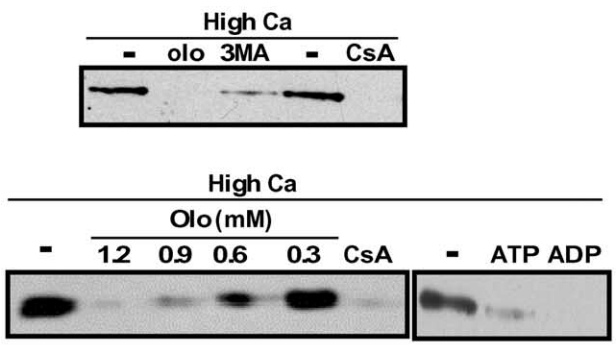
(B)


+PhAsO
A540
Time (min)
CsA
olo
3-MA
PhAsO

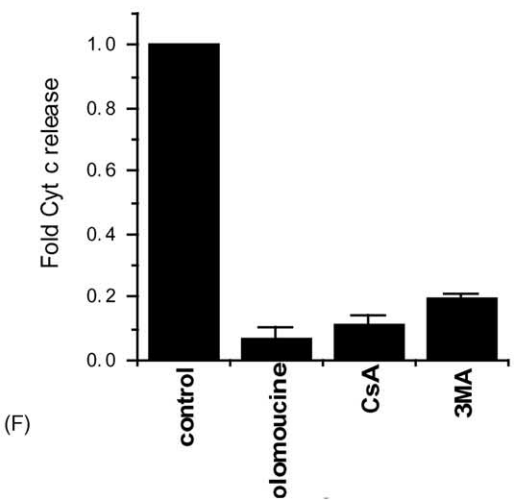
(C)


Swelling, %
[Olomoucine], mM
none
olo
iso-olo

(D)


High Ca
- olo 3MA - CsA
High Ca
Olo (mM)
- 1.2 0.9 0.6 0.3 CsA - ATP ADP

(E)


Fold Cyt c release
control
olomoucine
CsA
3MA

(F)

by adding 1 mM ADP. Uncoupled respiration was measured in the presence of 0.07 mM 2,4-dinitrophenol.

3. Results

3.1. Induction of mPT opening and cytochrome c release by araCTP and araC

In SCG neurones, addition of 0.1–1 mM concentrations of araC is required in order to induce apoptosis and this process takes several hours [6,31]. Because araC is metabolised rapidly to araCTP by cellular kinases, we examined the ability of both araC and araCTP to induce mPT at concentrations that were likely to be present in neuronal cells. When 1 mM araCTP was added to the mitochondria, it induced rapid mitochondrial swelling (Fig. 1A); the percentage of swelling after 10 min being 80% of the maximal rate of swelling induced by 55 μM $CaCl_2$ (Fig. 1B). The induction of mPT was dependent on the concentration of araCTP, but the dose dependence was not a simple function of concentration and showed both high- (ED_{50} ~50 μM) and low-affinity (ED_{50} ~0.5 mM) sites (Fig. 1C and D). The compound araC (1 mM) itself also induced swelling, but the extent of swelling was about 50% of that induced by 1 mM araCTP (Fig. 1A and B). In contrast, neither 1 mM dCTP (Fig. 1A and B) nor 1 mM CTP (data not shown) induced mitochondrial swelling.

The binding of CsA to mitochondrial cyclophilin D inhibits mPT, inhibition of mitochondrial swelling by CsA being a well-established indicator of mPT activity (reviewed in [32]). Swelling induced by araCTP was inhibited by addition of CsA indicating that swelling was dependent on mPT. Interestingly, ATP which inhibited mPT opening induced by $CaCl_2$ (see Fig. 2B) also blocked the induction of mPT by araCTP (data not shown).

To determine whether araCTP and araC promote the release of cytochrome *c*, mitochondrial suspensions were collected rapidly after treatment with the various inducers, spun, and an aliquot of the incubation medium was separated on SDS/PAGE and western blotted. Fig. 1E and F show that both araCTP and araC promoted the release of cytochrome *c* and that release induced by araCTP was inhibited by CsA, indicating that it was related to mPT.

3.2. Inhibition of mPT by 3-MA and olomoucine

Isolated liver mitochondria were treated such that they underwent very slow swelling by adding subthreshold levels of $CaCl_2$. Mitochondria were mixed with different concentrations of 3-MA, or CsA, in the presence of sub-optimal concentrations between 30 and 40 μM $CaCl_2$ in the presence of 0.48 mM KH_2PO_4 and induced to swell with another 10–15 μM $CaCl_2$ (55 μM final concentration). Fig. 2A shows that 3-MA inhibited mitochondrial swelling in a dose-dependent manner, with 1 mM 3-MA causing about 40% inhibition of swelling after 10 min compared to inhibition by CsA (considered as 100%). The inhibitory effect was dose dependent, but as much as 20 mM 3-MA were required to obtain 90% inhibition of swelling. Hence, 3-MA had relatively low efficacy as a complete inhibitor of the swelling process.

The effects of olomoucine (which inhibits apoptosis in primary neurones) were also investigated and compared with those of 3-MA, CsA, ATP and ADP (Fig. 2B). Olomoucine, but not its inactive analogue iso-olomoucine, inhibited swelling as effectively as CsA, ADP or ATP when it was added at a concentration of 1.2 mM, with half-maximal inhibition occurring at a concentration of about 0.4 mM (Fig. 2D). In keeping with these results, olomoucine also inhibited cytochrome *c* release in a dose-dependent manner (Fig. 2E and F), while the efficacy of 3-MA in inhibiting cytochrome *c* release was about 90% that of olomoucine or CsA (Fig. 2E and F). Inhibition of mPT opening by olomoucine and 3-MA was also provided when phenylarsine oxide was used as the mPT inducing agent (Fig. 2C), although the efficacy was reduced compared to CsA. The possibility that olomoucine and 3-MA might be inhibiting mPT by indirectly affecting respiration was examined by comparing its effects on state 3 respiration and uncoupled respiration with those of iso-olomoucine. Uncoupled respiration was reduced by 31 ± 7% by 1.2 mM olomoucine, 26 ± 1% by 1.2 mM iso-olomoucine and 14 ± 2% by 20 mM 3-MA. State 3 respiration was also inhibited by olomoucine (by 50 ± 6%), but once again this effect was also induced by iso-olomoucine (42 ± 1%) and to a lesser extent by 3-MA (14 ± 5%). Thus, it is unlikely that the effects of 3-MA and olomoucine on permeability transition are mediated entirely by their effects on respiration.

Fig. 2. 3-MA and olomoucine inhibit swelling induced by calcium or phenylarsine oxide. (A) Mitochondria were incubated without or with the indicated concentrations of 3-MA or 3.2 μM CsA (dashed line) in pre-swelling medium containing 0.48 mM KH_2PO_4 and 40 μM $CaCl_2$ and then induced to swell with another 15 μM $CaCl_2$ (high Ca). (B) Mitochondria were incubated in the presence of 1.2 mM olomoucine (olo), 5 mM ATP, 5 mM ADP, 20 mM 3-MA or 3.2 μM CsA, and then induced to swell with 0.48 mM KH_2PO_4 and 55 μM $CaCl_2$ (high Ca). Dashed line marked "low Ca" indicates mitochondria in pre-swelling medium (0.48 mM KH_2PO_4 and 37 μM $CaCl_2$). (C) Mitochondria were pre-incubated with 1.2 mM olomoucine (olo), 20 mM 3-MA or 3.2 μM CsA and then treated with phenylarsine oxide (PhAsO). (D) Dose response to olomoucine, with 100% swelling being the difference between swelling in the presence and absence of CsA. Inset shows results from two independent experiments which compare the effects of 0.9 or 1.2 mM olomoucine (olo) with those of 1.2 mM iso-olomoucine (iso-olo). (E) Cytochrome *c* release is inhibited by 3-MA and olomoucine. Upper panel, supernatants of mitochondria treated with 20 mM 3-MA, 1.2 mM olomoucine (olo) or 3.2 μM CsA. Bottom panel, supernatants of mitochondria shown in panels (B) and (D). (F) Quantification of cytochrome *c* release (1.2 mM olomoucine, 3.2 μM CsA, 20 mM 3-MA; mean ± range of two to three independent experiments). Equal input of mitochondria is indicated by the identical absorbance readings at time zero.

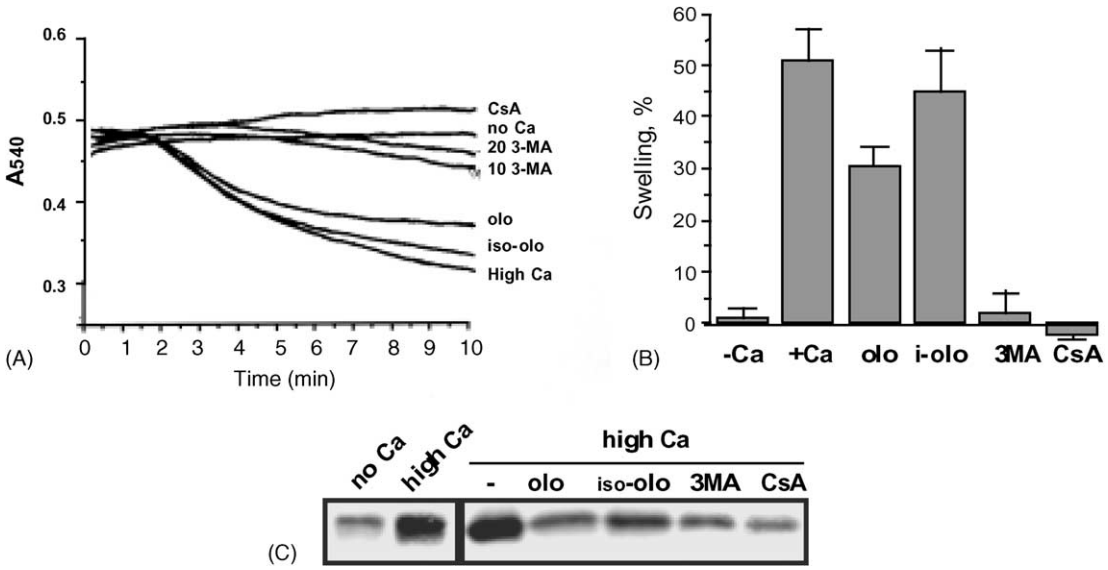


Fig. 3. 3-MA and olomoucine inhibit swelling of heart mitochondria. (A) Mitochondria were added last to swelling medium containing 0.1 mM $CaCl_2$ (high Ca) without or with one of the following drugs: 10 mM 3-MA, 20 mM 3-MA, 1 mM olomoucine (olo), 1 mM iso-olomoucine (iso-olo) and 1 μM CsA; "no Ca" indicates medium lacking $CaCl_2$. (B) Percent swelling was calculated by taking the difference in absorbance readings between 0 and 10 min, with 100% swelling being the difference between the values measured in the absence and presence of 0.1 mM $CaCl_2$. Results are mean ± range from two independent experiments. (C) Cytochrome *c* release: drugs concentrations are as indicated in panel (A), 3-MA was at 10 mM. Equal input of mitochondria is indicated by the similarity in absorbance readings at time zero.

3.3. Inhibition of mPT in heart mitochondria by olomoucine and 3-MA

To examine whether the inhibition of mPT by the adenine-based analogues extends to other types of mitochondria, heart mitochondria were induced to swell with Ca^{2+} and phosphate and the effects of olomoucine, iso-olomoucine and 3-MA were examined (Fig. 3). Heart mitochondria were chosen as they are well documented to contain a CsA-inhibitable mPT response that can be assayed *in vitro* [29], while brain mitochondria do not swell appropriately *in vitro* [33]. Although the swelling induced by calcium was less pronounced, the inhibitory responses to the drugs were still apparent. However, inhibition by 3-MA was relatively stronger in heart compared to liver mitochondria, while olomoucine inhibited less well, eliciting an inhibition of about 40%. There was also a slight inhibitory effect of iso-olomoucine.

4. Discussion

An increase in mitochondrial permeability is now established as an important route by which stimuli can activate apoptosis in mammalian cells. There is, therefore, much interest in finding drugs that can regulate the opening and closure of such permeability transitions in cancer and degenerative diseases, respectively. The ANT is an important component of mPT which is thought to constitute a pore that initially permits solutes of 1.5 kDa to permeate the inner mitochondrial membrane. Together with VDAC, it allows permeability of the outer membrane to much larger proteins (reviewed in [34]). The binding of ADP, dADP and ATP to ANT prevents activation of mPT induced by calcium loading or thiol oxidation reagents such as phenylarsine oxide [35]. In addition, CsA inhibits mPT pore activity by binding to cyclophilin D, a matrix protein that binds to ANT. Recently, it was shown that in contrast to ADP, dADP and ATP, which promote pore closure, addition of dATP to isolated mitochondria induced mPT pore opening and release of cytochrome *c* in a CsA-inhibitable manner. Cytochrome *c* release was sustained even when swelling was prevented by treatment with polyethylene glycol [2], indicating that the release of cytochrome *c* was not due entirely to non-specific membrane rupture. However, Halestrap *et al*. [35] have reported that a 1 mM concentration of other purines such as AMP, GTP, GDP and NAD derivatives have no inhibitory effect on calcium-induced mPT opening. Given these data, it is clear that the function of many nucleoside analogues that are being used in therapy in relation to mPT are impossible to predict from their structures alone. This difficulty is also observed when trying to account for the molecular actions of fluoro- and chloro-based deoxyadenosine analogues [3].

Pyrimidines are also used as anticancer and antiviral drugs [36,37], but hardly any work has been done on pyrimidine analogues in relation to mPT, although it is well established that at least human mitochondria have a dCTP-specific transporter [38]. Thus, we thought it reasonable to test whether some of the pyrimidine analogues that activate apoptosis may induce mPT while adenine-based nucleoside analogues, which inhibit neuronal apoptosis

and prevent cytochrome *c* release from the mitochondria, may prevent mPT opening.

We found that araC, but even more potently its active metabolite araCTP, induced swelling of isolated liver mitochondria in a CsA-inhibitable manner indicating induction of mPT opening. Swelling was accompanied by cytochrome *c* release which was also inhibited by CsA. The precise mechanism is still unclear. The mPT opening was unlikely to involve Bax activation since we found that there was no Bax in the membranes of these mitochondria, in keeping with the results of Polster *et al*. [39]. Since araCTP is a pyrimidine, it is not likely that it binds directly to the ADP-binding sites in ANT as these have strict steric requirements. It is also unlikely that the effect is mediated *via* the triphosphate moiety of the molecule as both araCTP and dCTP share the same triphosphate structure, yet the latter had no activity. Moreover, araC also had a toxic, although partial, effect. Although arabinose is a closer mimetic of 2′-deoxyribose compared to ribose, and dATP likewise induces mPT, dADP also lacks a 2′-hydroxyl group, yet it is a high-affinity inhibitor of ANT-dependent mPT, like ADP. Because of the rapidity of the response to araC and araCTP, it is not likely that the toxicity is associated with damage of mitochondrial DNA as this effect takes several days to manifest itself in cells and requires incorporation into mitochondrial DNA [36]. It is possible that araCTP and araC have a pro-oxidant effect [5,40], although the rapidity of response would suggest this is unlikely. Clearly, the precise mechanism of toxicity requires further study.

We also found that 3-MA, and more potently olomoucine, are capable of inhibiting calcium- (and phosphate)-induced swelling of liver mitochondria almost as well as ADP or ATP. Inhibition of cytochrome *c* release by olomoucine was as strong as that mediated by CsA. Because suppression of the respiratory chain can lead to a decrease in mitochondrial membrane potential and, hence, inhibition of calcium accumulation [41] which causes swelling, we tested the effects of the drugs on respiration. All three drugs had some inhibitory effects on respiration. As they inhibited both ADP-dependent (state 3) and uncoupled respiration, the inhibition was due to an effect on the electron transport chain rather than the phosphorylating subsystem. However, as iso-olomoucine inhibited respiration almost as potently as olomoucine, yet it had no effect on swelling, it is unlikely that inhibition of respiration is solely responsible for the inhibition of mPT and cytochrome *c* release.

The results with 3-MA support the view expressed by Lemasters and colleagues [22,23] that autophagic signals are emitted from mitochondria following mPT but the exact course of events is not known. Although 3-MA inhibits mPT with very low potency in the range of 5–20 mM, the dose response matches its efficacy in inhibiting autophagy (and apoptosis) in SCG neurones [20] and also its inhibition of JNK phosphorylation (we calculated an IC_{50} value of 6.5–7 mM for both effects). However, it is possible that in cells 3-methyl-adenosine is metabolised, for example, to 3-MA, which might also be more efficacious in inhibiting mPT. In keeping with this notion, olomoucine (an adenosine analogue which inhibits cyclin-dependent kinase 2 at low micromolar concentrations [24]) inhibited mPT with an ED_{50} of ~0.5 mM. This is about twice the concentration that inhibits apoptosis induced by trophic-factor deprivation in neurones (IC_{50} of ~200 μM in rat SCG neurones and several types of chick neurones [26]). Olomoucine also inhibits the activity of the stress kinase JNK with the same IC_{50} as it inhibits apoptosis, and this inhibition has been suggested to be the reason for its anti-apoptotic effects [26]. Our data suggest that, in addition, olomoucine might inhibit mPT, thereby sparing mitochondria from damage that leads to cytochrome *c* release and apoptotic death.

Since it has been shown that isolated brain mitochondria cannot be induced to swell by calcium in a manner that is inhibited solely by CsA [33,39,42]—although CsA is able to prevent cell death and mitochondrial swelling through regulation of mPT after ischaemia in the CNS [10] and in cultured neurones [43]—we used isolated heart mitochondria to examine whether we could generalise our observations. Damage to the heart following an ischaemic insult or nitric oxide treatment causes a CsA-inhibitable release of cytochrome *c* which can be assayed in isolated mitochondria [29]. We found that 3-MA completely inhibited swelling of heart mitochondria at 10 mM, while olomoucine (at 1 mM) inhibited swelling by 40%. Similarly, cytochrome *c* release was inhibited by both drugs. Excluding the simple explanation that the differences observed might be because of the different conditions required in the two swelling assays, these results suggest that mitochondria from different tissues possess both common and specific features. Nevertheless, it appears that assays on liver mitochondria can be used to screen for drugs that are likely to have similar effects on mitochondria from other tissues.

Preventing damage to mitochondria is of paramount importance for preservation of cell function in face of degenerative insults. In the nervous system, CsA treatment can provide protection against some forms of insult, but its use can be problematic. Given the direct effects on mPT of the cytidyl and adenine analogues used in this study, our observations not only suggest alternative mechanisms of actions for these well-known drugs, but also highlight the importance of screening for effects on mPT when designing drugs that induce, or prevent, cell death.

Acknowledgments

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References

[1] Costantini P, Jacotot E, Decaudin D, Kroemer G. Mitochondrion as a novel target of anticancer chemotherapy. J Natl Cancer Inst 2000;92:1042–53.

[2] Yang JC, Cortopassi GA. dATP causes specific release of cytochrome C from mitochondria. Biochem Biophys Res Commun 1998;250:454–7.

[3] Genini D, Adachi S, Chao Q, Rose DW, Carrera CJ, Cottam HB, Carson DA, Leoni LM. Deoxyadenosine analogs induce programmed cell death in chronic lymphocytic leukemia cells by damaging the DNA and by directly affecting the mitochondria. Blood 2000;96: 3537–43.

[4] Genini D, Budihardjo I, Plunkett W, Wang X, Carrera CJ, Cottam HB, Carson DA, Leoni LM. Nucleotide requirements for the in vitro activation of the apoptosis protein-activating factor-1-mediated caspase pathway. J Biol Chem 2000;275:29–34.

[5] Geller HM, Cheng KY, Goldsmith NK, Romero AA, Zhang AL, Morris EJ, Grandison L. Oxidative stress mediates neuronal DNA damage and apoptosis in response to cytosine arabinoside. J Neurochem 2001;78:265–75.

[6] Anderson CN, Tolkovsky AM. A role for MAPK/ERK in sympathetic neuron survival: protection against a p53-dependent, JNK-independent induction of apoptosis by cytosine arabinoside. J Neurosci 1999;19:664–73.

[7] Baker WJ, Royer Jr GL, Weiss RB. Cytarabine and neurologic toxicity. J Clin Oncol 1991;9:679–93.

[8] Sullivan PG, Thompson M, Scheff SW. Continuous infusion of cyclosporin A postinjury significantly ameliorates cortical damage following traumatic brain injury. Exp Neurol 2000;161:631–7.

[9] Shiga Y, Onodera H, Matsuo Y, Kogure K. Cyclosporin A protects against ischemia-reperfusion injury in the brain. Brain Res 1992;595: 145–8.

[10] Friberg H, Ferrand-Drake M, Bengtsson F, Halestrap AP, Wieloch T. Cyclosporin A, but not FK 506, protects mitochondria and neurons against hypoglycemic damage and implicates the mitochondrial permeability transition in cell death. J Neurosci 1998;18:5151–9.

[11] Shah AK. Cyclosporine A neurotoxicity among bone marrow transplant recipients. Clin Neuropharmacol 1999;22:67–73.

[12] Monteiro L, Almeida-Pinto J, Rocha N, Lopes G, Rocha J. Case report: cyclosporin A-induced neurotoxicity. Br J Radiol 1993;66:271–2.

[13] Trullemans F, Grignard F, Van Camp B, Schots R. Clinical findings and magnetic resonance imaging in severe cyclosporine-related neurotoxicity after allogeneic bone marrow transplantation. Eur J Haematol 2001;67:94–9.

[14] Gottrand F, Largilliere C, Farriaux JP. Cyclosporine neurotoxicity. N Engl J Med 1991;324:1744–5.

[15] Serkova N, Jacobsen W, Niemann CU, Litt L, Benet LZ, Leibfritz D, Christians U. Sirolimus, but not the structurally related RAD (everolimus), enhances the negative effects of cyclosporine on mitochondrial metabolism in the rat brain. Br J Pharmacol 2001;133:875–85.

[16] Tsuji A, Tamai I, Sakata A, Tenda Y, Terasaki T. Restricted transport of cyclosporin A across the blood–brain barrier by a multidrug transporter, P-glycoprotein. Biochem Pharmacol 1993;46:1096–9.

[17] Saito T, Zhang ZJ, Tokuriki M, Ohtsubo T, Shibamori Y, Yamamoto T, Saito H. Cyclosporin A inhibits the extrusion pump function of p-glycoprotein in the inner ear of mice treated with vinblastine and doxorubicin. Brain Res 2001;901:265–70.

[18] Seglen PO, Gordon PB. 3-Methyladenine: specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes. Proc Natl Acad Sci USA 1982;79:1889–92.

[19] Caro LH, Plomp PJ, Wolvetang EJ, Kerkhof C, Meijer AJ. 3-Methyladenine, an inhibitor of autophagy, has multiple effects on metabolism. Eur J Biochem 1988;175:325–9.

[20] Xue L, Fletcher GC, Tolkovsky AM. Autophagy is activated by apoptotic signalling in sympathetic neurons: an alternative mechanism of death execution. Mol Cell Neurosci 1999;14:180–98.

[21] Jia L, Dourmashkin RR, Allen PD, Gray AB, Newland AC, Kelsey SM. Inhibition of autophagy abrogates tumour necrosis factor alpha induced apoptosis in human T-lymphoblastic leukaemic cells. Br J Haematol 1997;98:673–85.

[22] Lemasters JJ, Nieminen AL, Qian T, Trost LC, Elmore SP, Nishimura Y, Crowe RA, Cascio WE, Bradham CA, Brenner DA, Herman B. The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy. Biochim Biophys Acta 1998;1366:177–96.

[23] Elmore SP, Qian T, Grissom SF, Lemasters JJ. The mitochondrial permeability transition initiates autophagy in rat hepatocytes. FASEB J 2001;17:17.

[24] Vesely J, Havlicek L, Strnad M, Blow JJ, Donella-Deana A, Pinna L, Letham DS, Kato J, Detivaud L, Leclerc S. Inhibition of cyclin-dependent kinases by purine analogues. Eur J Biochem 1994;224: 771–86.

[25] Maas Jr JW, Horstmann S, Borasio GD, Anneser JM, Shooter EM, Kahle PJ. Apoptosis of central and peripheral neurons can be prevented with cyclin-dependent kinase/mitogen-activated protein kinase inhibitors. J Neurochem 1998;70:1401–10.

[26] Markus MA, Kahle PJ, Winkler A, Horstmann S, Anneser JM, Borasio GD. Survival-promoting activity of inhibitors of cyclin-dependent kinases on primary neurons correlates with inhibition of c-Jun kinase-1. Neurobiol Dis 1997;4:122–33.

[27] Di Lisa F, Menabo R, Canton M, Barile M, Bernardi P. Opening of the mitochondrial permeability transition pore causes depletion of mitochondrial and cytosolic NAD+ and is a causative event in the death of myocytes in postischemic reperfusion of the heart. J Biol Chem 2001;276:2571–5.

[28] McBride AG, Borutaite V, Brown GC. Superoxide dismutase and hydrogen peroxide cause rapid nitric oxide breakdown, peroxynitrite production and subsequent cell death. Biochim Biophys Acta 1999;1454:275–88.

[29] Borutaite V, Morkuniene R, Brown GC. Release of cytochrome c from heart mitochondria is induced by high Ca^{2+} and peroxynitrite and is responsible for Ca(2+)-induced inhibition of substrate oxidation. Biochim Biophys Acta 1999;1453:41–8.

[30] Tedeschi H, Harris DL. Some observations on the photometric estimation of mitochondrial volume. Biochim Biophys Acta 1958;28: 392–402.

[31] Martin DP, Wallace TL, Johnson Jr EM. Cytosine arabinoside kills postmitotic neurons in a fashion resembling trophic factor deprivation: evidence that a deoxycytidine-dependent process may be required for nerve growth factor signal transduction. J Neurosci 1990;10:184–93.

[32] Vieira HL, Haouzi D, El Hamel C, Jacotot E, Belzacq AS, Brenner C, Kroemer G. Permeabilization of the mitochondrial inner membrane during apoptosis: impact of the adenine nucleotide translocator. Cell Death Differ 2000;7:1146–54.

[33] Andreyev AY, Fahy B, Fiskum G. Cytochrome c release from brain mitochondria is independent of the mitochondrial permeability transition. FEBS Lett 1998;439:373–6.

[34] Crompton M. Mitochondrial intermembrane junctional complexes and their role in cell death. J Physiol 2000;529:11–21.

[35] Halestrap AP, Woodfield KY, Connern CP. Oxidative stress, thiol reagents, and membrane potential modulate the mitochondrial permeability transition by affecting nucleotide binding to the adenine nucleotide translocase. J Biol Chem 1997;272:3346–54.

[36] Rossi L, Serafini S, Schiavano GF, Casabianca A, Vallanti G, Chiarantini L, Magnani M. Metabolism, mitochondrial uptake and toxicity of 2′,3′-dideoxycytidine. Biochem J 1999;344:915–20.

[37] Feng JY, Johnson AA, Johnson KA, Anderson KS. Insights into the molecular mechanism of mitochondrial toxicity by AIDS drugs. J Biol Chem 2001;276:23832–7.

[38] Bridges EG, Jiang Z, Cheng YC. Characterization of a dCTP transport activity reconstituted from human mitochondria. J Biol Chem 1999; 274:4620–5.

[39] Polster BM, Kinnally KW, Fiskum G. BH3 death domain peptide induces cell type-selective mitochondrial outer membrane permeability. J Biol Chem 2001;276:37887–94.

[40] Iacobini M, Menichelli A, Palumbo G, Multari G, Werner B, Del Principe D. Involvement of oxygen radicals in cytarabine-induced apoptosis in human polymorphonuclear cells. Biochem Pharmacol 2001;61:1033–40.

[41] Gunter KK, Gunter TE. Transport of calcium by mitochondria. J Bioenerg Biomembr 1994;26:471–85.

[42] Brustovetsky N, Brustovetsky T, Jemmerson R, Dubinsky JM. Calcium-induced cytochrome c release from CNS mitochondria is associated with the permeability transition and rupture of the outer membrane. J Neurochem 2002;80:207–18.

[43] Khaspekov L, Friberg H, Halestrap A, Viktorov I, Wieloch T. Cyclosporin A and its nonimmunosuppressive analogue N-Me-Val-4-cyclosporin A mitigate glucose/oxygen deprivation-induced damage to rat cultured hippocampal neurons. Eur J Neurosci 1999;11:3194–8.