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The CDK inhibitor, R-roscovitine, promotes eosinophil apoptosis by down-regulation of Mcl-1

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ABSTRACT

Eosinophils are major players in inflammatory allergic diseases such as asthma, hay fever and eczema. Here we show that the cyclin-dependent kinase inhibitor (CDKi) R-roscovitine efficiently and rapidly induces human eosinophil apoptosis using flow cytometric analysis of annexin-V/propidium iodide staining, morphological analysis by light microscopy, transmission electron microscopy and Western immunoblotting for caspase-3 cleavage. We further dissect these observations by demonstrating that eosinophils treated with R-roscovitine lose mitochondrial membrane potential and the key survival protein Mcl-1 is down-regulated. This novel finding of efficacious induction of eosinophil apoptosis by CDKi drugs has potential as a strategy for driving resolution of eosinophilic inflammation.

1. Introduction

The eosinophil is a leukocyte of granulocyte lineage that confers resistance to parasitic infection [1]. In some societies levels of parasitic infection have significantly declined and in parallel, allergic, eosinophil-mediated, inflammatory diseases such as asthma, eczema and hay-fever have increased. In direct contrast, in those countries where parasitic infection has not declined, allergic inflammatory diseases are not prominent. It seems as though functional redundancy is not an option for eosinophils and allergic disease results from their inappropriate activation [2,3]. Eosinophils and their products have been demonstrated within airways, in lung parenchyma, at the site of eczematous skin lesions and in nasal mucosa. Evidence of their armamentarium has also been detected at sites of allergic inflammation and it has been shown that they contribute to airway epithelial damage and remodelling in asthma [1,4–7]. Therapeutic strategies that decrease eosinophil recruitment and activation or enhance resolution of inflammation by driving eosinophil apoptosis and clearance should ameliorate allergic inflammatory disease [1,3,8].

An obvious target for the prevention of eosinophil growth, differentiation, survival [9–11] and, also to some extent recruitment and activation [12] is the cytokine IL-5 but initial in vivo work has been disappointing suggesting a subordinate role or even redundancy for eosinophils in asthma [13]. Despite this, eosinophils have retained their place as key pathophysiological players in defined subsets of asthma following successful trials of anti-IL-5 therapy [14–16]. If eosinophils can be driven towards apoptosis and efficiently cleared by phagocytes prior to membrane rupture, the damaging sequelae of degranulation and reactive oxygen species leakage should be avoided, thus protecting airway tissue and preventing detrimental remodelling [5,17]. Eosinophils undergo classical apoptosis in response to various treatments including: NF-κB inhibition, Fas-ligation, protein synthesis inhibition and exposure to corticosteroids. There is some controversy over the dominant mechanism by which eosinophils undergo apoptosis. Eosinophils are known to possess mitochondria which are important for apoptosis but probably not functional in terms of ATP

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generation. Caspases 3, 8 and 9 are constitutively expressed and have all been in implicated in dexamethasone driven apoptosis [7,18–20].

The cyclin-dependent kinase inhibitor (CDKi), R-roscovitine, was initially developed as an anti-cancer agent directed against cell-cycle proteins, promoting cancer cell death through effects on NF-κB, p53 and key survival proteins [21,22]. It has been shown to promote apoptosis of myeloid cancer cells by down-regulation of the key survival protein Mcl-1 [23]. Eosinophils are terminally differentiated cells and as such should not require cell-cycling machinery such as CDKs. Nonetheless, like other terminally differentiated cells, including neutrophils [24,25] and neurons [26], they have measurable expression of CDKs. Neutrophils are known to undergo apoptosis in response to CDK inhibitors and indeed work performed in our laboratory has shown that resolution of neutrophil-driven inflammation can be driven by this class of drugs [27].

In this paper, we are the first to show that CDK inhibition drives eosinophil apoptosis by the mitochondrial pathway, a novel finding with promising implications for the resolution of eosinophilic inflammation.

2. Materials and methods

2.1. Eosinophil isolation

Granulocytes were isolated from the peripheral venous blood of healthy adult donors (Lothian Research Ethics Committee approvals #08/S1103/38 or #1702/95/4/72) by dextran (Pharmacosmos) sedimentation followed by centrifugation through discontinuous PBS-Percoll (GE Healthcare) gradients [28,29]. Eosinophils were separated from contaminating neutrophils using an immunomagnetic separation step with sheep anti-mouse IgG-Dynabeads (Invitrogen) coated with the murine anti-neutrophil antibody 3G8 (anti-CD16; a gift from Dr. J. Unkeless, Mount Sinai Medical School, New York) as previously described [30]. Eosinophil purity was routinely greater than 95%.

2.2. Apoptosis assessment

Eosinophils were re-suspended in IMDM (PAA) with 10% FBS (Biosera), penicillin (100 U/ml) and streptomycin (100 U/ml) (PAA). Cells were aliquoted (5×10^6 cells/ml) into a 96-well-flat-bottomed-flexible-plate (BD Biosciences) in a final volume of 150 μl. They were incubated with R-roscovitine (A.G. Scientific, USA), Z-Val-Ala-DL-Asp(OMe)-fluoromethylketone (zVAD-fmk) (Bachem, Switzerland) or combinations at 37 °C with 5% CO_2. All stock reagents were initially dissolved in dimethylsulphoxide (DMSO) (Sigma) then diluted in buffer yielding a final DMSO concentration of 0.2%; a corresponding DMSO control of 0.2% was assessed as an appropriate vehicle control. We assessed apoptosis by flow cytometry using annexin-V-FLUOS (Roche) in combination with propidium iodide (PI) (Sigma) on a BD FacsScan flow cytometer (Becton, Dickinson and Co.). Morphological apoptotic changes were assessed by light microscopy of DiffQuik™ (Dade Behring) stained cytocentrifuged cells.

2.3. Mitochondrial transmembrane potential assessment

MitoCapture™ is a fluorescence-based tool for distinguishing between viable and apoptotic cells by detecting changes in the mitochondrial transmembrane potential. Eosinophils were isolated as above and incubated with appropriate reagent at a concentration of 5×10^4 ml^{-1} in a flat-bottomed 96-well plate at 37 °C, 5% CO_2. MitoCapture™ kit used as per manufacturer's instructions. Fluorescence microscopy was performed using a Zeiss Axiovert S100 microscope.

2.4. Western blotting

Cells were incubated in 2 ml Eppendorf tubes at a concentration of 5×10^6 cells/ml with R-roscovitine, zVAD-fmk, MG-132 (Calbiochem) or combinations of these reagents for 4 h at 37 °C. Cells were lysed using 1% IgepalCA-630 (Sigma) in TBS containing a protease inhibitor cocktail before centrifugation (23 100×*g*; 4 °C; 20 min) [24]. Protein samples (equivalent to 1.5×10^6 cells/lane) were resolved by SDS–PAGE (12% gel Thermo Scientific) then transferred to PVDF membranes (Millipore). Blots were blocked with 5% skimmed milk powder in TBS/0.1% Tween-20 (Sigma) before probing with antibodies to Mcl-1 (Santa Cruz), caspase-3 (BD Biosciences), cleaved caspase-3 (Cell Signaling Technologies) and soluble β-actin (Sigma).

2.5. Electron microscopy

Cells were incubated with R-roscovitine (20 μM) at 37 °C and 5% CO_2 for 8 h. Fixation was achieved by resuspension in 3% gluteraldehyde/0.1 M sodium cacodylate buffer (pH 7.4) overnight. Photomicrographs were taken with a Phillips CM12 transmission electron microscope.

2.6. Statistical methods

Statistics shown are analysis of variance with post hoc testing by Student–Newman–Keuls using InStat software and data are expressed as means ± S.E.M. unless otherwise stated.

3. Results

3.1. R-roscovitine can drive primary human eosinophil apoptosis in a concentration- and time-dependent manner

To explore whether R-roscovitine promotes similar pro-apoptotic effects on human eosinophils as those previously observed with neutrophils, cells were treated with increasing concentrations of 10, 20 and 50 μM R-roscovitine. Apoptosis was assessed at time points as early as 4 h by flow cytometric analysis and R-roscovitine was observed to induce apoptotic cell death in eosinophils. This contrasts with other pro-apoptotic agents such as dexamethasone where apoptosis is first detected at 12–24 h [30,31]. In an early, preliminary experiment we included dexamethasone (1 μM) as a positive control for induction of eosinophil apoptosis. We found that R-roscovitine (30 μM) was more effective at inducing apoptosis as assessed by annexin-V positivity. Interestingly, a combination of the two agonists did not yield higher levels of cell death than R-roscovitine alone. For example, after 20 h culture, control death was 12.5 ± 0.8%; dexamethasone was 22.1 ± 2.2%; R-roscovitine was 81.2 ± 1.1% and a combination of dexamethasone and R-roscovitine was 83.9 ± 1.4% where values from duplicate samples obtained from a single donor are expressed as the means ± S.D. Cells were further assessed for any morphological changes by light microscopy after cytocentrifugation and DiffQuik™ staining. The results showed a clear increase in annexin-V/propidium iodide staining, which occurred in a concentration- and time-dependent manner (Figs. 1A, B and 2A). Eosinophils progress through apoptosis to secondary necrosis (shown by joint staining with annexin-V and propidium iodide). This transition appears to occur more rapidly in eosinophils than in the other abundant granulocyte, neutrophils. At later time-points (up to 20 h) we see increasing levels of apoptosis and a concomitant rise in necrosis again suggesting transition from apoptosis to secondary necrosis. Transmission electron microscopy of R-roscovitine treated eosinophils (20 μM, 8 h) was utilised to ultimately characterise their cellular morphology. These

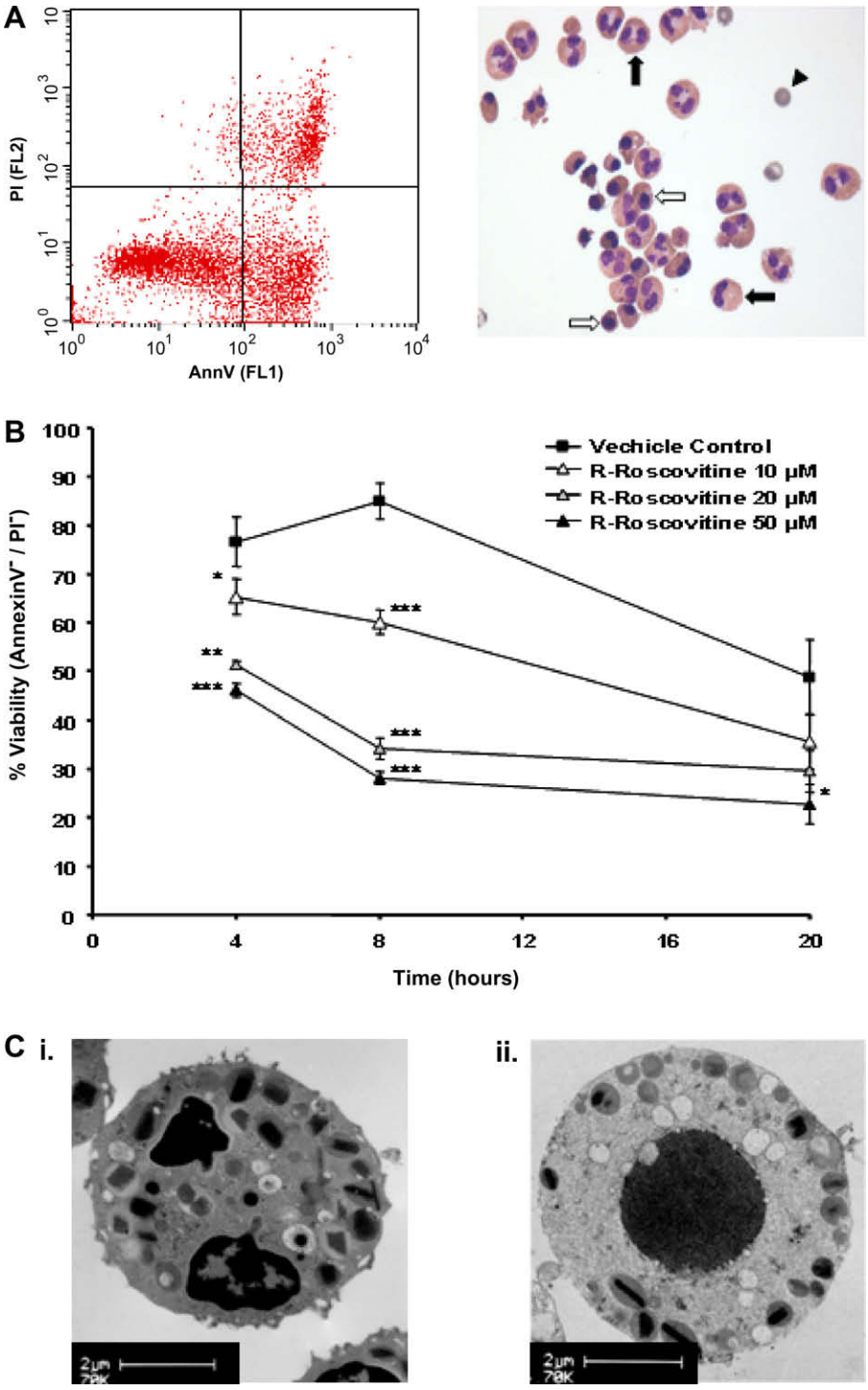

Fig. 1. Ability of increasing concentrations (10, 20 and 50 μM) of R-roscovitine, to induce eosinophil cell death after 4 h treatment. (A) Flow cytometric profile of annexin-V/PI staining showing apoptotic cells in the lower right quadrant, necrotic cells in the top right quadrant and viable cells in the bottom left quadrant. Cytocentrifuge image (400× magnification) demonstrates cellular morphology. Black arrows indicate healthy, viable eosinophils and white arrows indicate apoptotic eosinophils. Black arrow head indicating an erythrocyte. (B) Cumulative flow cytometric analysis of the mean percentage annexin-V^-/PI^- cells. There were significant differences (using analysis of variance and post hoc Student–Newman–Keuls) between each treatment at the relevant time-point, P values displayed are $^{*}P \leqslant 0.05$, $^{**}P \leqslant 0.01$, $^{***}P \leqslant 0.001$ and represent differences in levels of apoptosis against the DMSO control, $n = 3$ independent experiments. (C) Transmission electron microscopy images of (i) a healthy non-apoptotic human eosinophil and (ii) an apoptotic eosinophil 8 h post-R-roscovitine (20 μM) treatment. The latter cell is exhibiting characteristics typical of apoptosis, such as a condensed, round nucleus. Magnification 9500×.

results clearly demonstrate the presence of classical apoptotic signs; namely the presence of chromatin condensation in the nucleus and granular rearrangement, when comparing R-roscovitine treated apoptotic eosinophils (Fig. 1C(ii)) with non-apoptotic cells (Fig. 1C(i)).

3.2. R-roscovitine driven eosinophil apoptosis can be delayed by caspase inhibition

Classical apoptosis is known to be a caspase-dependent phenomenon [32,33]. In order to confirm our finding that eosinophils

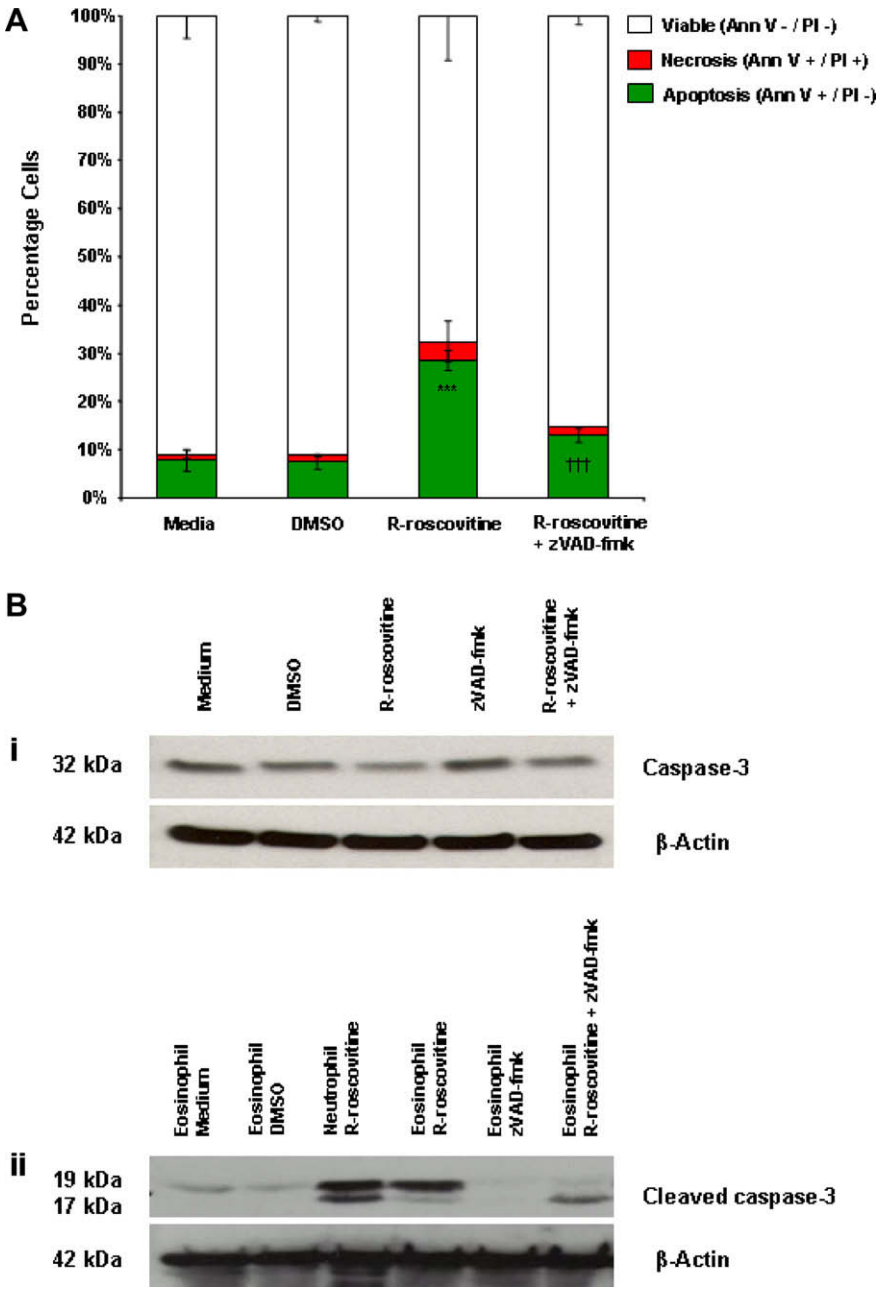

Fig. 2. Apoptosis induced 4 h post-treatment with R-roscovitine (50 μM) was inhibited by zVAD-fmk (100 μM), indicating that the mechanism of cell death is caspase-dependent. (A) Cumulative flow cytometric profile analysis showing the mean percentage of eosinophils in the viable (white bar), necrotic (red bar) or apoptotic (green bar) quadrants. *P* values (using analysis of variance and post hoc Student–Newman–Keuls) of $^{***}P \leqslant 0.001$ represent difference in apoptosis compared to controls and *P* values $^{\dagger\dagger\dagger}P \leqslant 0.001$ are compared to R-roscovitine treatment, $n = 4$ independent experiments. (B) (i) Representative Western immunoblot image for caspase-3 of the lysates from eosinophils treated with either R-roscovitine (50 μM) alone or a co-treatment of R-roscovitine and zVAD-fmk (100 μM) for 4 h. (ii) Representative Western immunoblot image for cleaved caspase-3 of the lysates from eosinophils treated with either R-roscovitine (50 μM) alone or a co-treatment of R-roscovitine and zVAD-fmk (100 μM) for 4 h. Also included are the lysates of eosinophils treated with DMSO (0.2%) as a vehicle control, culture medium alone, zVAD-fmk alone and lysates of R-roscovitine-treated human neutrophils (identical conditions) as a positive control. A β-actin blot is also included as a loading control.

undergo apoptosis following incubation with CDKis, we assessed caspase involvement using two different approaches. Initially, we co-incubated eosinophils with R-roscovitine (50 μM) and zVAD-fmk (100 μM), the pan-caspase inhibitor and measured apoptosis, as before, using flow cytometry following annexin-V and propidium iodide staining. Co-incubation appeared to inhibit the early induction of apoptosis. At 4 h the level of apoptosis decreased significantly ($P \leqslant 0.001$) by over 50% from 27.3 ± 2.0% to 13.1 ± 1.5% (Fig. 2A). To further demonstrate the role of caspases in R-roscovitine induced eosinophil apoptosis, we also performed Western blotting for caspase-3 (Fig. 2B(i)) and cleaved caspase-3 (Fig. 2B(ii)) on lysates from eosinophils, 4 h post-R-roscovitine with and without zVAD-fmk treatment. There were changes in the levels of caspase-3 on R-roscovitine and zVAD-fmk treatment as detected by a specific caspase-3 antibody and no evidence of cleaved caspase-3 isoforms were detected with this antibody. These changes correlated with our subsequent finding using a specific cleaved caspase-3 antibody. Cleaved casapase-3 was easily detected in cells after R-roscovitine treatment with the use of a specific cleaved caspase-3 antibody. However, co-treatment with R-roscovitine and zVAD-fmk reduced caspase-3 cleavage (Fig. 2B(ii)) thereby confirming the caspase-dependent nature of

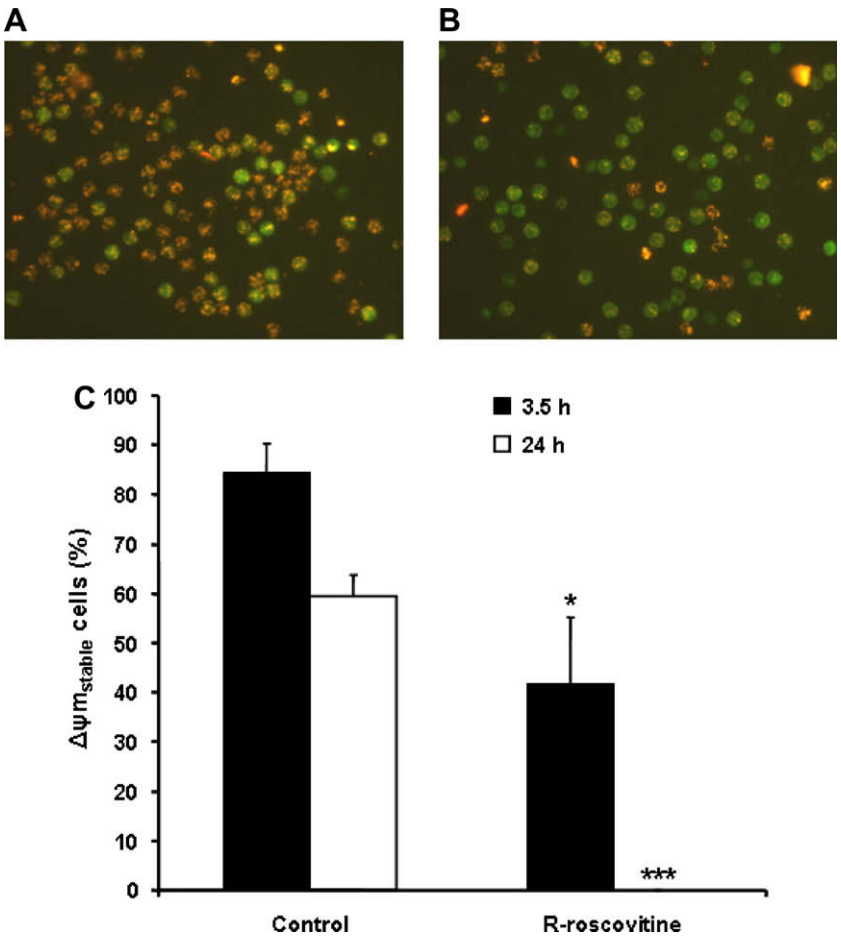

Fig. 3. Assessment of mitochondrial membrane potential using the MitoCapture™ assay. (A) Eosinophils cultured in media supplemented with FCS at 37 °C, 5% CO_2 for 3.5 h were assayed. The vast majority of eosinophils retain mitochondrial membrane potential ($\Delta\psi_m$ stable) as identified by orange/red fluorescence. Representative images were taken 320× magnification. (B) Eosinophils cultured as above, however treated with R-roscovitine, 20 μM for 3.5 h. R-roscovitine treatment results in a significant increase in the proportion of eosinophils losing mitochondrial membrane potential as identified by green fluorescence within the cytoplasm. (C) Quantification of immunofluorescence showing the percentage cells in each mitochondrial membrane stage at both 3.5 and 24 h. *P* values (using analysis of variance and post hoc Student–Newman–Keuls) of $^{*}P \leqslant 0.05$ control vs R-roscovitine 3.5 h and $^{***}P \leqslant 0.001$, control vs R-roscovitine 24 h are displayed. $n = 3$ independent experiments with >500 cells counted per condition.

R-roscovitine induced cell death. Interestingly, despite an overall decrease in cleaved caspase-3 levels with combined R-roscovitine and zVAD-fmk treatment there was a slight increase in the levels of the 17 kDa cleaved caspase-3 isoform. (The visible bands at MW 17 and 19 kDa are cleaved caspase-3 isoforms (Fig. 2B(ii), inactive (non-cleaved) caspase-3 is evident at 32 kDa (Fig. 2B(i)).

3.3. Apoptosis promoted by CDK inhibition is mediated by loss of mitochondrial membrane potential

In order to further investigate our finding that eosinophils undergo apoptosis following treatment with R-roscovitine (20 μM) we assessed mitochondrial membrane potential with the use of the mitochondria specific dye, MitoCapture™. This dye stains viable mitochondria orange/red as visualised by fluorescence microscopy, but dissipates into the cell cytoplasm and fluoresces green with loss of membrane potential. Loss of mitochondrial membrane potential ($\Delta\psi_m$) is a key event in the intrinsic pathway of apoptosis and we were able to show that significantly more eosinophils lost mitochondrial membrane potential at 3.5 h post-R-roscovitine treatment compared to control (Fig. 3). At 24 h post-R-roscovitine treatment no eosinophils had intact mitochondria, whereas the majority of control eosinophils retained mitochondrial membrane potential (Fig. 3C).

3.4. R-roscovitine down-regulates Mcl-1, a key eosinophil survival protein

Having found that eosinophil mitochondrial membrane potential was lost following treatment with R-roscovitine, we postulated that this might be due to the down-regulation of key survival proteins. The Bcl-2 homologue, Mcl-1, has a short half-life because it can be ubiquitinated and degraded in the proteasome. It has previously been shown in neutrophil studies that treatment with MG-132, a proteasome inhibitor, preserves Mcl-1 and prevents apoptosis at early time-points [30]. At late time-points other effects such as the preservation of IκBα lead to acceleration of apoptosis [34]. Eosinophils co-incubated with both MG-132 (50 μM) and R-roscovitine (50 μM) for 4 h had enhanced viability compared to those treated with R-roscovitine alone (48.7 ± 10.7% annexin-V^-/PI^- vs 29.9 ± 10.1% annexin-V^-/PI^- $P \leqslant 0.05$, $n = 5$). We demonstrated that this MG-132 survival effect correlated with preservation of Mcl-1 protein in contrast to eosinophils incubated with R-roscovitine alone where Mcl-1 was significantly down-regulated (Fig. 4). This contrasted with cells co-incubated with R-roscovitine and zVAD-fmk (100 μM) where no preservation of Mcl-1 was apparent (to be expected as zVAD-fmk inhibits caspases which would normally function downstream of Mcl-1 down-regulation).

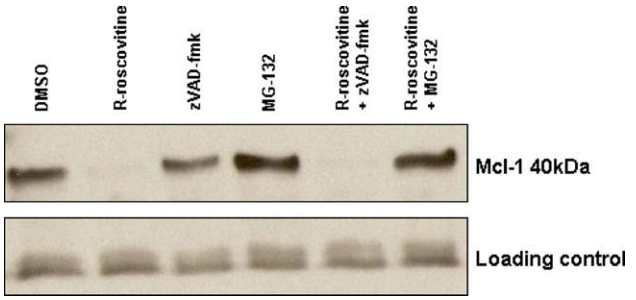

Fig. 4. Western immunoblotting of human eosinophil lysates for Mcl-1 (40 kDa) demonstrating significant down-regulation following 4 h R-roscovitine (20 μM) treatment. The proteasome inhibitor, MG-132 (50 μM) prevented R-roscovitine mediated down-regulation of Mcl-1 whilst the caspase inhibitor, zVAD-fmk (100 μM), did not. Loading control is a non-specific band at 20 kDa on the same gel.

4. Discussion

We present the novel finding of rapid and efficacious induction of human eosinophil apoptosis, in vitro, with the CDKi drug, R-roscovitine. This is an important finding as eosinophils play a central role in the pathophysiology of common allergic diseases such as asthma and hay-fever as well as uncommon diseases such as eosinophilic oesophagitis, eosinophilic pneumonia and Churg-Strauss syndrome [1,10,35]. Eosinophils are also notoriously difficult to study for a number of reasons: their paucity in the peripheral blood of normal, healthy volunteer blood donors, the laborious isolation techniques required to ensure a pure population and their relative intractability to standard molecular biological techniques such as siRNA. Corticosteroids are known to induce significant eosinophil apoptosis, in vitro, but it is still debatable as to whether this effect is relevant in vivo [6,31,36–40]. Steroid drugs are extremely effective in the majority of asthmatic patients but some, at the more severe end of the spectrum, are resistant and others experience intolerable and unacceptable side-effects [41–45]. The initial disappointing results with therapy attempting to neutralise IL-5 had suggested that eosinophils were perhaps innocent bystanders in the disease process [13]. Two recent positive trials using the same approach with a carefully selected eosinophilic population of asthmatic patients suggest that eosinophils are central to a sub-population of asthmatic disease in what is increasingly recognised to be a heterogeneous patient group [14–16,45].

We have published results indicating that neutrophil apoptosis can be driven by CDKi drugs and that this can in turn promote resolution of inflammatory disease models [27]. We now show that this same drug-class can promote eosinophil apoptosis and we dissect the molecular mechanism by which this occurs. The mechanisms by which apoptosis occur in eosinophils remain controversial but it seems likely that the general paradigm of extrinsic (death-receptor mediated) and intrinsic (mitochondria mediated) pathways is applicable [18,19,46,47]. We have shown that eosinophils undergo apoptosis at early time-points (4 h) following treatment with CDKi and that this is preceded by loss of the key survival protein Mcl-1. Mcl-1 is now generally accepted to be the pre-eminent Bcl-2 homologue responsible for neutrophil survival and evidence is increasing for a central role in eosinophil survival. This evidence includes the granulopenic phenotype of the *Mcl* $1^{-/-}$ mouse [48] and the finding that dexamethasone (a prototype corticosteroid) driven eosinophil apoptosis is associated with down-regulation of Mcl-1 at the protein level [39]. These pro-survival Bcl-2 homologues are short-lived compared to their pro-apoptotic counterparts, a feature which presumably biases granulocyte cells towards early, constitutive apoptosis. This is not the case in other cell types as, for example, the eponymous Bcl-2 protein has a much longer half-life and therefore might be expected to protect cells from short-lived interruptions to survival protein production [49]. This may explain why we see little effect of CDK inhibition on macrophage survival, a finding that protects our paradigm of therapeutic, selective apoptosis induction followed by efficient phagocytic clearance.

In keeping with our results suggesting down-regulation of survival proteins, we also show an early (3.5 h) loss of mitochondrial membrane potential. This immediately precedes significant caspase-3 cleavage and supports our contention that we are inducing classical apoptosis mediated via the mitochondrial pathway as opposed to direct activation of the death-receptor pathway. We further support our data regarding caspase involvement, using the pan caspase inhibitor, zVAD-fmk at a concentration of 100 μM. This drug has been used over a concentration range of 10–300 μM in the literature and often at 100 μM in granulocyte work [47,48]. At 300 μM in combination with TNF accelerated apoptosis has been observed [49]. It is our experience working with primary human granulocytes that conventional low-dose zVAD-fmk is rapidly degraded by these cells. The capacity of granulocytes for zVAD-fmk degradation over short time-periods can lead to the erroneous conclusion that apoptosis, especially constitutive apoptosis, is caspase independent. Therefore, by necessity, we are forced to use higher concentrations than those routinely used by researchers working with other cell types. We are aware that non-specific effects on calpains have been described with caspase inhibitors but we feel that this pharmacological observation, together with the Western blotting analysis of caspase cleavage, strongly indicate that CDK inhibitor-induced apoptosis is indeed caspase-dependent. We have further demonstrated that the induction of apoptosis mediated by CDK inhibition is more powerful than that observed with dexamethasone. We are inclined to believe, given the data presented, that CDK inhibition mediates a more effective down-regulation of survival proteins. As CDK inhibition will promote neutrophil apoptosis in tandem with eosinophil apoptosis there is a risk of inducing neutropenia. There is evidence to suggest that activated neutrophils are preferentially targeted by CDKi (which may confer additional benefit in some asthma patients) but a strategy involving dual therapy with antibiotics might also be envisaged. The potential of this approach has been demonstrated in a recent paper by Koedel et al. [50] where combined CDKi and ceftriaxone therapy is used to resolve an experimental model of bacterial meningitis. Our novel finding, that CDKi drugs drive eosinophil apoptosis suggests that this approach may prove an effective resolution strategy for eosinophilic inflammation.

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