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Letter to the Editor

Fenretinide induces cytochrome *c* release, caspase 9 activation and apoptosis in the absence of mitochondrial membrane depolarisation

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Dear Editor,

Among the retinoids, all-*trans*-*N*-(4-hydroxyphenyl)retinamide (fenretinide) is particularly promising as an antitumour agent as it has been reported to have fewer negative effects than naturally occurring retinoids such as all-*trans* retinoic acid (ATRA) and can induce cell death (by apoptosis) even in ATRA-resistant cell lines.[1]

Although fenretinide is thought to induce apoptosis through a p53-independent pathway, it is not clear whether the biological effects are mediated by a direct interaction with nuclear retinoid receptors or via novel nuclear receptor-independent pathways.[2,3] There are at least two (and possibly three) distinct major pathways in the initiation of apoptosis.[4] One of these pathways, thought to be triggered by chemotherapeutic drugs, involves the release of cytochrome *c* from the mitochondria to the cytosol, where it forms an essential part of the vertebrate 'apoptosome', composed of cytochrome *c*, Apaf-1 and procaspase 9. The apoptosome triggers the ATP-dependent activation of caspase 9, which then processes and activates other downstream caspases to orchestrate the biochemical execution of cells.

Cytochrome *c* release is often accompanied by a reduction in mitochondrial membrane potential ($\Delta\psi_m$). Indeed, a reduction in $\Delta\psi_m$ and the resultant depolarisation of mitochondria have been proposed as ubiquitous events in the apoptotic programme although this assertion has been challenged by recent studies. The molecular effects of fenretinide are controversial in this respect and the role of mitochondria are particularly contentious. Both Lovat *et al*.[5] and Suzuki *et al*.[6] observed that fenretinide triggers cytochrome *c* release but disagreed about the effects on mitochondrial membrane depolarisation. In the present report, we found that fenretinide did not disrupt mitochondrial transmembrane potential but did trigger cytochrome *c* release from the mitochondria into the cytosol. In accord with earlier reports our data suggest that dissipation of the mitochondrial transmembrane potential may be dispensable for apoptosis[5,7] and that cytochrome *c* can be released regardless of the disruption of the membrane potential.[5,8] Indeed, in a separate study we have found that apoptosis can proceed in the absence of both cytochrome *c* release and mitochondrial transmembrane depolarisation.[9]

In the present study we investigated the cytotoxic effects of fenretinide on A431 epidermoid carcinoma cells to further dissect the mechanism of cell death at the molecular level. We first measured the effect of fenretinide on the survival of A431 cells using morphological analysis and the metabolic MTT assay. Cells were treated with a range of concentrations (0.1–10 μM) of fenretinide, 500 μM hydrogen peroxide (H_2O_2), 1 μg/ml cycloheximide or vehicle alone (0.1% v/v DMSO) for 48 h. Metabolic analysis by MTT assay, indicated that fenretinide induced cell death in a dose-dependent manner (Figure 1a). For both fenretinide and cycloheximide these effects were more pronounced in low serum (0.5%), while H_2O_2 was equally toxic in both 10 and 0.5% serum.

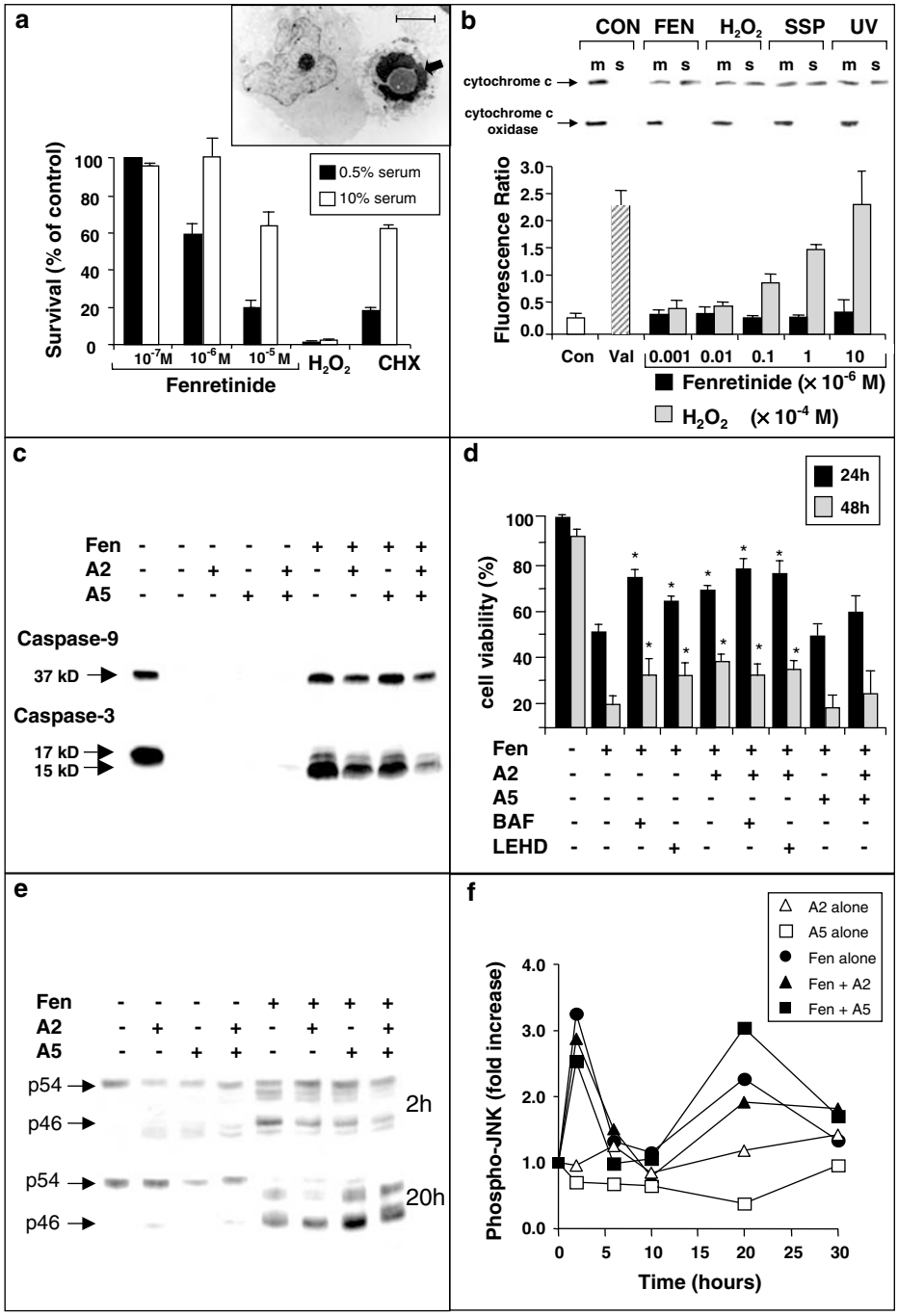
The mode of cell death induced by fenretinide was identified as apoptosis by electron microscopy. Following exposure to fenretinide (10 μM for 48 h), a number of ultrastructural changes were observed in A431 cells consistent with apoptotic cell death, including plasma membrane blebbing, cytoplasmic shrinkage and chromatin condensation (Figure 1a, inset). Apoptosis was also confirmed by DNA fragmentation detected by the TUNEL assay; fenretinide treatment resulted in a seven-fold increase in the number of TUNEL-positive cells from 7.2% in control cultures to 53.6% in those exposed to fenretinide (not shown).

To detect the release of cytochrome *c* into the cytosol, Western blotting was performed on subcellular fractions of A431 cells treated for 48 h either with 10 μM fenretinide, 500 μM H_2O_2, 1 μM staurosporine (SSP) or UV irradiation (a 30 min exposure to 700 mW cm^{-2} UVB). In this series of experiments, cytochrome oxidase (a resident protein of the inner mitochondrial membrane) was used as a marker for contamination of cytoplasmic fractions with mitochondrial components. As shown in Figure 1b (top panel), both cytochrome oxidase and cytochrome *c* were absent from the cytosolic fractions of control cultures. In contrast, exposure of A431 cells to fenretinide, H_2O_2, SSP or UVB significantly increased cytochrome *c* levels in the cytosolic fraction, while cytochrome *c* oxidase was retained in the mitochondria of the same cells.

To determine whether cytochrome *c* release was associated with a disruption of mitochondrial transmembrane potential, A431 cell cultures were loaded with the cationic fluorochrome JC-1, the aggregation of which depends on the mitochondrial transmembrane potential.[10] The fluorescence was measured at emission wavelengths specific for JC-1 monomers (530 nm) or aggregates (590 nm). In control cultures, the ratio of JC-1 monomers to aggregates was less than 0.5, reflecting the baseline for healthy mitochondria in A431 cells. Following an 8 h exposure to valinomycin (a positive control for mitochondrial depolarisation) or H_2O_2 the ratio increased to approximately 2.5, indicating mitochondrial

membrane depolarisation. In contrast, fenretinide did not induce significant changes in mitochondrial transmembrane potential (Figure 1b, bottom panel). Similar results were observed in time-course experiments to detect any transient changes following exposure to fenretinide (data not shown).

Since cytochrome *c* release occurred in the absence of changes in mitochondrial potential, it was not obvious that fenretinide-induced apoptosis proceeded through a classical caspase-dependent mitochondrial pathway. We therefore investigated caspase 9 and caspase 3 processing, by Western blotting using cleavage-specific antibodies. As shown in Figure 1c there was no detectable caspase 9 or 3 processing in untreated A431 cells. However, in cultures exposed to fenretinide, there was clear evidence of processing of both caspases. Cleavage of caspase 9 was not affected by the inclusion of A5, an RARα-specific antagonist, but was significantly lower in the presence of an RARβ/γ-specific antagonist A2. The effect of A2 on fenretinide-induced caspase 3 cleavage was less pronounced, although the combination of A2 and A5 significantly reduced the amount of



cleaved products of both caspases. These data indicate that caspase activation following exposure to fenretinide is partially mediated through RARs.

These observations led us to question whether RAR antagonists would protect against fenretinide-induced apoptosis and so we determined the viability of A431 cells after treatment with fenretinide in the presence or absence of the two RAR antagonists or defined caspase inhibitors. Fenretinide alone caused a 50% reduction in MTT metabolism after 24 h and this fell further to 80% of healthy cultures after 48 h. Only the RARβ/γ-specific antagonist A2 protected against fenretinide cytotoxicity, although the rescue was only partial (Figure 1d). Similarly, both the pan-caspase inhibitor Boc-aspartyl-FMK (BAF) or the more selective caspase 9 inhibitor LEHD-FMK also partially protected against fenretinide-induced cell death, increasing cell viability at both 24 and 48 h. The protective effects of BAF and LEHD-FMK were increased further in the presence of the RARβ/γ-specific antagonist A2, although the combination of caspase inhibitor and RAR antagonist still did not result in complete rescue from fenretinide-induced apoptosis. Indeed, a significant amount (20%) of fenretinide-induced cell death was still observed in the combined presence of A2 and BAF. These results suggest that fenretinide can trigger apoptosis partly through a RAR- and caspase-independent pathway.

Since the toxic effects of fenretinide could not be completely blocked by a combination of caspase inhibitors and RAR antagonists, it was important to further investigate the signalling pathways triggered by this retinoic acid derivative. We focused on Jun N-terminal kinase (JNK) phosphorylation (an indirect measure of activation) in A431 cells, since there are conflicting reports in the literature about the differential effects of retinoids on JNK activity associated with cell death.[11,12] While novel synthetic retinoids have been shown to activate JNK/c-jun signalling leading to apoptosis in cancer cells,[11] in other cell types retinoic acid has been implicated in antiapoptotic processes associated with JNK inhibition.[12] In the present study, phosphorylation of both the p54 and p46 isoforms of JNK increased significantly in A431 cells treated with fenretinide (Figure 1e). In a kinetic analysis, it was apparent that this increase was biphasic, reaching a maximum of three-fold above controls at 2 h, declining to basal levels and then increasing again to two-fold above controls at 20 h (Figure 1f). The RARβ/γ-specific antagonist A2, but not the α-antagonist A5, reduced the early peak in fenretinide-dependent JNK phosphorylation by around 30% but had no significant effect at later times. It is therefore likely that the secondary rise in JNK phosphorylation is a consequential response in cells already committed to apoptotic death, particularly since this occurs after caspase 3 activation, which peaks at 10 h after fenretinide exposure (data not shown). While these observations suggest that JNK activation may not be significant in the apoptotic response of A431 cells to fenretinide, we measured the total levels of phospho-JNK and so cannot exclude the possibility that one of the (10) isoforms plays a more specific role.

In summary, we have demonstrated that fenretinide-induced apoptosis in A431 cells is accompanied by cytochrome *c* release, but occurrs independently of mitochondrial membrane depolarisation. Fenretinide signalling is complex and although JNK, caspase 9 and caspase 3 are all activated, the combination of RAR antagonists and caspase inhibitors are only partially protective. Our results suggest that fenretinide-induced apoptosis is complex and proceeds through both RAR-dependent and -independent pathways. A challenge of the future will be to unravel this complexity in order to identify missing components of the fenretinide death machinery.

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Figure 1 (**a**) Effect of fenretinide on MTT metabolism. A431cells (15 000 per well) were seeded in 96-well plates overnight and then treated with defined concentrations of fenretinide, hydrogen peroxide (H_2O_2, 500 μM), or cycloheximide (CHX, 1 μg/ml) for 48 h. Cytotoxicity was assessed by the MTT assay,[9] in which the number of living cells is proportional to the reduction of a colorimetric substrate, measured by the absorbance at 562 nm. Data represent the mean of six wells (mean $\pm$ S.D.). (**a**) Subcellular changes in fenretinide-treated A431 cells. Healthy cells were allowed to adhere to circular cover slips in 24-well plates overnight and then treated with 10 μM fenretinide for a further 48 h. At the end of the experiment, cell monolayers were fixed and examined by electron microscopy. Condensed chromatin and nuclear pyknosis (bold arrow) associated with cytoplasmic shrinkage can be seen in the apoptotic cell, but are absent from the healthy cell on the left (bar=5 μm). (**b**) top: Effect of fenretinide on cytochrome *c* release. A431 cells were seeded in 10-cm tissue culture dishes overnight and then treated with fenretinide (10 μM), H_2O_2 (500 μM) or staurosporine (1 μM) for 48 h or exposed to UVB radiation for 30 min followed by a further 48 h incubation. Cells were fractionated and analysed for cytochrome *c* and cytochrome oxidase expression by immunoblotting.[9] M, mitochondrial fraction; S, supernatant (cytosolic fraction); CON, control; FEN, fenretinide (1 μM); H_2O_2, hydrogen peroxide (100 μM); SSP, staurosporine (500 nM); UV, ultra-violet B irradiation (30 min). (**b**) bottom: Effect of fenretinide on mitochondrial transmembrane potential. A431cells (15 000 per well) were seeded in 96-well plates overnight and then treated with defined concentrations of fenretinide or H_2O_2 for 8 h. Valinomycin (1 μM) was used as a positive control. Changes in the mitochondrial transmembrane potential were measured using the indicator dye, JC-1.[10] Data represent the mean values ($\pm$ S.D.) from six separate wells. (**c**) Fenretinide activation of caspases. A431 cells were treated for 10 h with 1 μM fenretinide (Fen) and 1 μM retinoid acid receptor alpha (RARα) antagonist, CD503 (A5) or RARβ/γ, CD2665 (A2). Cell lysates were probed in immunoblotting experiments with specific antibodies recognising cleaved caspase 3 (p15 and p17) or cleaved caspase 9 (p37) fragments. Cell lysates from staurosporine-treated SHSY5Y cells were used as a positive control, lane 1). (**d**) Differential effects of RAR antagonists and caspase inhibitors on fenretinide cytotoxicity. A431cells were seeded in 96-well plates (15 000 per well) overnight and then cultured for a further 24 or 48 h in the presence or absence of 1 μM fenretinide (Fen), 1 μM retinoid acid receptor alpha (RARα) antagonist, CD-503 (A5) or RARβ/γ, CD2665 (A2). In some experiments, A431 cells were pre-treated for 30 min with the pan-caspase inhibitor BOC-aspartyl-FMK (50 μM) or specific caspase 9 inhibitor z-LEHD-FMK (20 μM). Cell viability was determined by the MTT assay and the data represent the mean ($\pm$ S.D.) of 6 wells; $^*P<0.05$ compared to wells treated with fenretinide alone. (**e**) Effects of fenretinide JNK phosphorylation. A431 cells were treated for the indicated times with 1 μM fenretinide (Fen) alone or in the presence of 1 μM retinoid acid receptor alpha (RARα) antagonist, CD503 (A5) or RARβ/γ, CD2665 (A2). Cell lysates were probed in immunoblotting experiments with a phosphospecific JNK antibody recognising two bands (p46 and p54) corresponding to active JNK isoforms (from Cell Signaling Technology, MA, USA) and visualised by enhanced chemiluminescence. (**f**) The resulting blots from two separate experiments, measuring total JNK phosphorylation (i.e the sum of p46 and p54) over a 30-h period, were scanned by densitometry and the data obtained plotted graphically

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