The impact of retinoic acid treatment on the sensitivity of neuroblastoma cells to fenretinide

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Abstract. Despite the successful introduction of 13-*cis* retinoic acid (13cisRA) therapy for the treatment of neuroblastoma, approximately 50% patients do not respond or experience relapse. A retinoid analogue, fenretinide [N-(4-hydroxyphenyl) retinamide; 4-HPR] can induce apoptosis in neuroblastoma cell lines and could have clinical use after therapy with 13cisRA. However, there are important questions concerning potential retinoid drug interactions which need to be addressed. The aim of this study was to investigate the influence of retinoic acid pre-treatment on fenretinide-induced apoptosis and fenretinide metabolism in neuroblastoma cell lines. Apoptosis was measured by flow cytometry of propidium iodide-stained neuroblastoma cells and a live-cell imaging assay. Intracellular fenretinide metabolism was determined by HPLC analysis. Pre-treatment of neuroblastoma cell lines with retinoic acid (RA) resulted in a significant decrease in the apoptotic response to fenretinide in three of the four lines tested. Comparison between responsive and non-responsive cell lines suggested that RA sensitivity was required to promote fenretinide resistance, and that this was mediated by up-regulation of Bcl-2 and the inhibition of pro-apoptotic fenretinide signalling pathways. Induction of the oxidative metabolism of fenretinide after RA pre-treatment did not significantly impact on intracellular parent drug levels and is unlikely to explain the decreased apoptotic response observed. The interaction between RA and fenretinide could have important implications for the scheduling of fenretinide in therapeutic protocols for neuroblastoma.

Introduction

Neuroblastoma is the leading cause of cancer deaths in children aged 1-4 years. Arguably the most significant development in the treatment of neuroblastoma over the past 10 years has been the addition of 13-*cis* retinoic acid (13cisRA) for the treatment of high-risk disease, which, in clinical trials, has resulted in a 17% increase in 3-year event-free survival (EFS) when administered after intensive chemotherapy and myeloablative therapy (1,2). Although approximately 50% patients either do not respond or relapse after 13cisRA therapy, the substantial increase in EFS to date demonstrates the value of using retinoids in the context of minimal residual disease (MRD). Recent work both in Europe and America has raised the prospect that a retinoid analogue, fenretinide [N-(4-hydroxyphenyl) retinamide; 4-HPR], could have a significant impact on therapeutic strategies for neuroblastoma (3,4). Fenretinide can induce growth arrest and apoptosis in a variety of tumour types including neuroblastoma, and has generated considerable interest because of its mild toxicity and favourable pharmacokinetic profile in paediatric patients. Furthermore, prolonged disease stabilisation was induced in some neuroblastoma patients participating in a phase I trial when treated during partial remission (3). These encouraging clinical data, together with the observation that neuroblastoma cells shown to be resistant to 13cisRA are sensitive to fenretinide (5), have led to the incorporation of fenretinide in the treatment of MRD in a phase II trial. However, it is known that retinoic acid (RA) treatment can increase resistance to apoptotic agents (6-8), and whilst combining fenretinide with 13cisRA represents a promising clinical approach, there are important questions over the potential for interactions between these drugs which have yet to be addressed. Indeed, a recent paper by Hadjidaniel and Reynolds has highlighted the antagonistic effects of 13cisRA on the cytotoxic potential of chemotherapeutic agents including etoposide, topotecan and doxorubicin in a panel of neuroblastoma cell lines (9). In terms of potential interactions between RA treatment and fenretinide, it is important to consider the effect of RA on factors involved in the mechanism of action of fenretinide as well as enzymes involved in fenretinide metabolism. For example, induction of RA hydroxylase CYP26 has been shown to increase the oxidative metabolism and decrease the efficacy of retinoid drugs (10).

Fenretinide-induced apoptosis is mediated by the up-regulation of pro-apoptotic factors GADD153 and Bak, followed by cytochrome c release, caspase activation and apoptosis as a

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result of oxidative and endoplasmic reticulum (ER) stress (11). ER stress is a recently discovered novel aspect of fenretinide action (12), and work from our group highlights the role of the pro-apoptotic BH3-only branch of the Bcl-2 family of proteins in this context (13). As such, it is likely that expression of pro-survival members of the Bcl-2 family will impact on sensitivity to fenretinide. A previous study describing the potential importance of Bcl-2 and Bcl-xL up-regulation during neuronal differentiation and subsequent resistance to apoptosis is therefore of particular relevance (7). Conversely, neuroblastoma cells treated intermittently with a high concentration of all-*trans* RA (ATRA) *in vitro* to select for cells resistant to differentiation were hypersensitive to fenretinide (5). These data indicate that cells sensitive to RA (able to differentiate in response to RA) may be resistant to subsequent treatment with agents that induce apoptosis, while cells unable to respond to RA would remain sensitive. As neuroblastoma is notoriously heterogeneous, cells exhibiting these properties may exist as subpopulations within a tumour and influence response to therapy. Analysis of fenretinide cytotoxicity in a panel of neuroblastoma cell lines which were established from patients at various phases of therapy, including myeloablative therapy and 13cisRA treatment, revealed a range of sensitivities to fenretinide, with resistance more apparent post-treatment (5). Therefore, 13cisRA therapy may influence response to subsequent treatment with fenretinide in subsets of patients. Furthermore, since RA therapy results in the induction of RA metabolism via CYP26, 13cisRA therapy may increase the metabolism of fenretinide with deleterious consequences for therapeutic efficacy. The aim of the current study was to test the hypothesis that pre-treatment of neuroblastoma cells with retinoic acid can negatively impact on the ability of fenretinide to induce apoptosis. Insights into potential mechanisms which may elucidate such an observation were obtained through investigation of Bcl-2 family protein expression and the effect of RA-induced CYP26 expression on fenretinide intracellular metabolism.

Materials and methods

Cell lines. SH-SY5Y, SKNBE(2c), NGP and IMR-32 neuroblastoma cell lines were cultured routinely in RPMI-1640 medium containing foetal calf serum (10%). Cells were grown at 37˚C in a humidified incubator containing 5% CO_2.

Drug treatment. 13cisRA and ATRA (Sigma) were dissolved in DMSO, fenretinide (Janssen-Cilag Ltd., Basserdorf, Switzerland) dissolved in ethanol and N^6,2'-O-Dibutyryladenosine 3',5'-cyclic monophosphate (db-cAMP: Sigma) dissolved in water, prior to dilution in cell culture medium. The final concentration of vehicle in all cases was <0.2%. All experiments were performed in dim light and tubes containing retinoids were wrapped in aluminium foil.

Analysis of cell death. Flow cytometry of propidium-iodide-stained cells was used to estimate the increase in sub-G1 content as a measure of cell death as previously described (13). In addition, cell death was determined by a microscopy-based assay simultaneously imaging caspase 3 activity and propidium iodide accumulation on individual cells every hour for 12 h. Cell death was determined by the accumulation of propidium iodide in the nucleus and caspase 3 activity was imaged with NucView™ 488 (Biotium, Hayward, USA). Both parameters were expressed as the percentage of all nuclei at each time-point as determined by Hoechst 33258 (Sigma, Poole, UK) accumulation. NucView 488 is a cell permeable caspase 3 DEVD substrate coupled to a fluorescent DNA dye. The substrate is not fluorescent but caspase 3 cleavage yields a product that binds to DNA and fluoresces at 488ex/520em, thus generating an archive of caspase 3 activity within each cell over the observation period.

SH-SY5Y cells were plated at 1,000 cells/well in black-walled μ-clear bottom optical 96-well plates (Greiner, Gloucestershire, UK). On the day of treatment media were aspirated and replaced with 50 μl fresh media and 50 μl media containing Hoechst 33258 to a final concentration of 10 μg/ml 1 h before imaging, 50 μl media containing propidium iodide and NucView 488 caspase 3 substrate both to final concentrations of 1 μM 15 min before imaging and 50 μl media containing fenretinide to a final concentration of 10 μM or the equivalent volume of vehicle immediately prior to imaging. Three wells were also stained with Hoechst, NucView 488 or propidium iodide alone to determine exposure, gain and offset values that detect signal without optical bleed-through. Each well containing cells was imaged at 1 h intervals for 12 h on a Pathway HT automated fluorescent microscope (BD Biosciences, Oxford, UK) in wide field mode at 37˚C and 5% CO_2. Filter sets were: NucView 488, excitation 488/10 and emission 515LP, Hoechst, excitation 360/10 and emission 435LP and propidium iodide, excitation 548/20 and emission 570LP. A x20 objective lens was used and each image collected comprised a 2x2 montage of immediately adjacent fields of view.

Western blot analysis. Total protein was extracted from cell pellets and separated by electrophoresis through 4-20% SDS-PAGE gels (20 μg per track) and blotted onto PVDF membranes. Blots were probed with antibodies to Noxa (Alexis Biochemicals), GADD153 (clone B3), Mcl-1 (clone S-19, Santa Cruz Biotechnology, Heidelberg, Germany), Bcl-xL, Bcl-2 (New England Biolabs Ltd., Hitchin, UK) and, as a loading control, β-actin (Sigma). For detection, secondary peroxidase-conjugated antibodies (Millipore, Watford, UK) were employed and visualized using the ECL system (GE Healthcare UK Ltd., Buckinghamshire, UK).

Intracellular fenretinide metabolism and CYP26 induction. SH-SY5Y cells were plated at 20,000 cells/well in 6-well plates and allowed to establish growth over 24 h. Cells were pre-incubated with ATRA (10 μM) for 24 h, prior to aspiration of media and incubation of cells with media containing fenretinide (20 μM) for a period of 6 h. Treatment media was removed and cells were rinsed with ice-cold PBS prior to collection of cell pellets. Extraction of cell pellets was carried out with the addition of acetonitrile (100 μl), followed by vortexing and centrifugation at 15,000g for 5 min. The supernatant obtained was used for analysis of fenretinide and metabolite levels by HPLC analysis as previously described (14). Briefly, this involved separation of fenretinide and metabolites using a Waters Symmetry C18 3.5 μm column (4.6x150 mm) and a linear gradient over a 20-min run time between mobile phase A

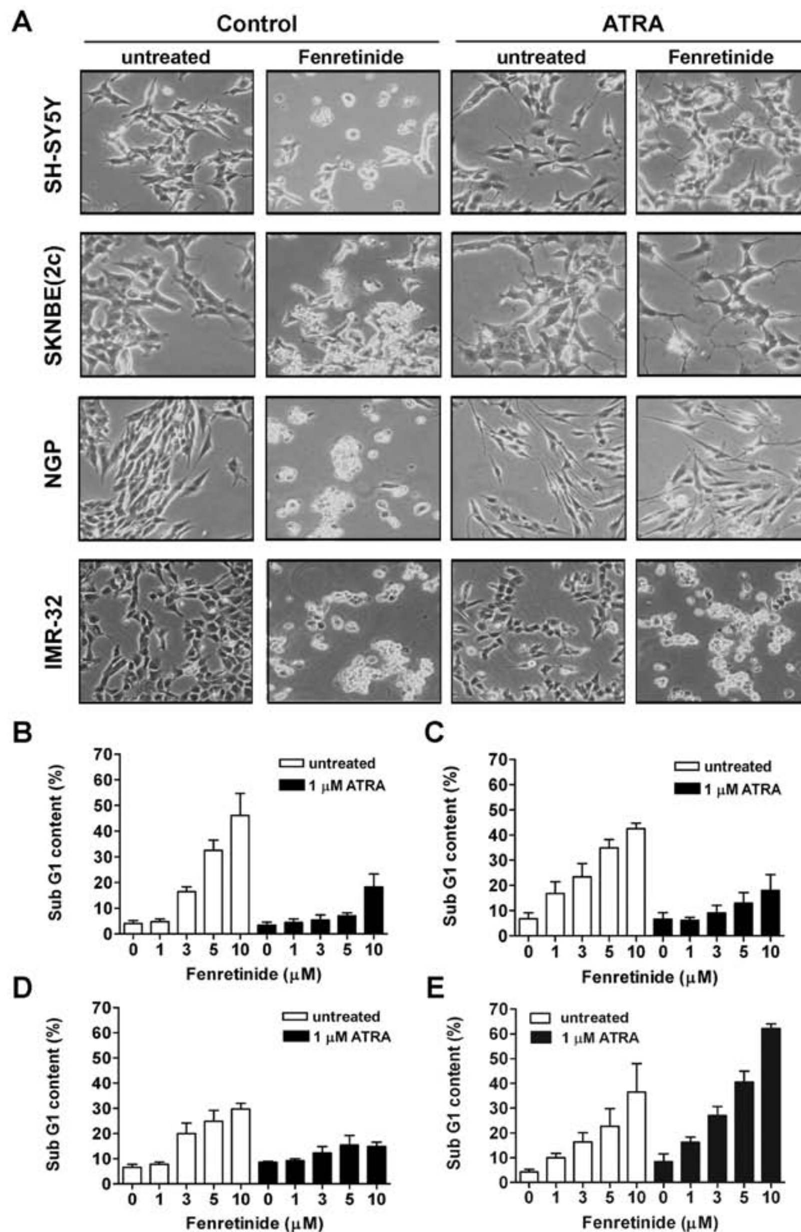

Figure 1. Cytotoxicity of fenretinide in neuroblastoma cells after RA treatment. SH-SY5Y (A, B), SKNBE(2c) (A, C), NGP (A, D) and IMR-32 (A, E) cells were pre-treated with ATRA (1 μM) for 5 days prior to washout and treatment with fenretinide [(A), 10 μM; (B-E), 1-10 μM] for a further 24 h. (A) Cells were visualised under phase contrast light microscopy or (B-E) cell death measured by flow cytometry of propidium iodide stained cells to determine the sub-G1 fraction. Data are expressed as percentage total population, where each point is the mean ± SEM, n ≥3.

[70% acetonitrile/30% acetic acid (0.2%)] and mobile phase B (acetonitrile/0.2% acetic acid). Analysis was carried out using a Waters 2690 Separations Module and 996 Photodiode array (PDA) detector (Waters Ltd., Elstree, UK). Determination of CYP26 expression levels was carried out as previously described (10). Briefly, RNA was extracted from cell pellets using the RNeasy® mini kit (Qiagen, Crawley, UK) and reverse transcribed using Promega's Reverse Transcription System according to the manufacturer's instructions. Real-time PCR was performed on 20 ng cDNA using TaqMan® Assays-on-Demand Gene Expression products for human CYP26A1 in combination with the TaqMan Universal PCR master mix (Applied Biosystems, Warrington, UK). Appropriate controls for non-specific amplification and contamination were included. A GeneAmp 5700 Sequence Detection System was used for real-time PCR amplification and data were analyzed using GeneAmp Sequence Detection System software.

Statistical analysis. Data were analysed using GLM and one-way ANOVA procedures in SPSS version 15 (SPSS Inc., Chicago, IL). Where appropriate, data were log-transformed to equalise variances and treatments compared using contrasts.

Results

Sensitivity of RA-treated neuroblastoma cells to fenretinide-induced cell death. To investigate the effects of retinoic acid treatment on sensitivity to fenretinide-induced apoptosis, a

panel of neuroblastoma cells, representing MYCN-amplified, p53 wild-type (NGP, IMR-32), MYCN-amplified, p53-mutated [SKNBE(2c)] and non-MYCN-amplified, p53 wild-type cells (SH-SY5Y), were treated with 1 μM ATRA for 5 days prior to increasing concentrations of fenretinide for a further 24 h. The differentiating properties of retinoic acid are well documented for neuroblastoma cells (15). In this study, cellular response to 1 μM ATRA was evaluated by morphological differentiation, characterised by neurite extension (Fig. 1A), and inhibition of cell proliferation (mean ± SEM relative to control, vehicle-treated cells: SH-SY5Y 56.02±4.55%, SKNBE(2c) 67.08±1.1%, NGP 67.27±3.9%, IMR-32 46.53±0.5%). In response to fenretinide, cell death increased in a concentration-dependent manner in all cell lines studied, however, after pre-treatment with 1 μM ATRA, cell death in response to fenretinide (1-10 μM) was significantly reduced compared to that in untreated cells in SH-SY5Y, SKNBE and NGP cells (GLM: ATRA main effect $F_{1,>20}$>9, P≤0.005; Fig. 1B-D). In marked contrast, ATRA pre-treatment of IMR-32 cells did not result in observable neurite outgrowth and increased the level of fenretinide-induced cell death (GLM: ATRA main effect $F_{1,16}$=11.8, P=0.003; Fig. 1E).

To assess whether the effects of retinoic acid were specific to the all-trans isomer, SH-SY5Y cells were pre-treated with 1 μM 13cisRA, 1 μM ATRA, 50 μM db-cAMP or control vehicle for 5 days prior to fenretinide for a further 24 h. Db-cAMP has been demonstrated to be a weak inducer of neuronal differentiation at this concentration (16) and was included to test the effects of a non-retinoid differentiation inducer. In the absence of fenretinide there was no difference in the level of apoptosis between pre-treatments ($F_{3,15}$=1.1, P=0.38); fenretinide induced apoptosis substantially in the vehicle-pre-treated cells and cells pre-treated with 50 μM db-cAMP (contrasts comparing response to fenretinide within ANOVA: $|t_{30}|$>6.8, P<0.001) but did not induce apoptosis in cells pre-treated with ATRA or 13cisRA [t_{30}=-1.9, P=0.063 for ATRA pre-treated cells and t_{30}=-0.08.9, P=0.94 for ATRA pre-treated cells (Fig. 2A)].

Similar results were obtained using a live cell imaging assay to detect caspase 3 activity. In control cells, caspase activity (Fig. 2B) and PI positive nuclei (data not shown) accumulated over time to 31 and 15%, respectively of all cells at 12 h in response to the addition of fenretinide. Pre-treatment of cells with db-cAMP partially inhibited the caspase 3 activity observed after fenretinide treatment (P=0.0001, Two-way ANOVA of curve compared with caspase activity of control cells), but not PI accumulation. In contrast, cells treated with 13cisRA or ATRA had <5% caspase- or PI-positive cells at 12 h (Fig. 2B). In both control and db-cAMP/RA cell populations in the absence of fenretinide <5% of cells were apoptotic at all time-points (data not shown). Propidium iodide accumulation lagged behind detectable caspase activity in all cells.

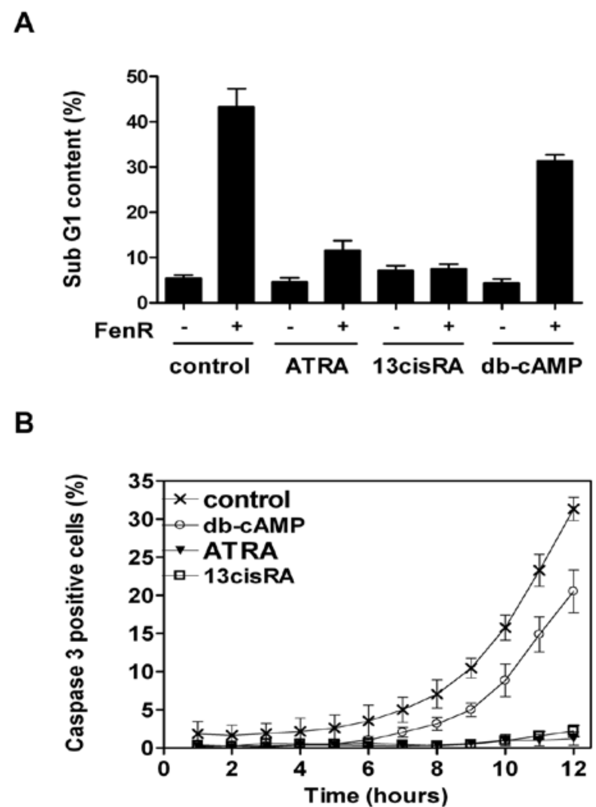

Figure 2. Apoptotic activity of fenretinide during SH-SY5Y differentiation. Cells were pre-treated with db-cAMP (50 μM), ATRA or 13cisRA (1 μM) for 5 days prior to washout and treatment with fenretinide (10 μM) for 24 h (A) or 0-12 h (B). (A) Cell death was measured by flow cytometry of propidium iodide stained cells to determine the sub-G1 fraction. Data are expressed as percentage total population, where each point is the mean ± SEM, n ≥3. (B) Caspase 3 activity was measured in live SHSY5Y cells and cells imaged as described in Materials and methods. Values represent mean of 3 or 2 (for cAMP values) replicates over 2 independent experiments (mean ± SEM, n ≥2).

Impact of RA on fenretinide signalling. The ability of fenretinide to induce an ER stress response and subsequent activation of Noxa and cell death suggests that RA-induced expression of pro-survival members of the Bcl-2 family may mediate resistance to fenretinide-induced apoptosis (12,13). The expression of Bcl-2 family proteins was therefore evaluated in SH-SY5Y and IMR-32 cells, which acquired fenretinide-resistance or remained fenretinide-sensitive after ATRA treatment respectively (Fig. 3). ATRA treatment resulted in markedly increased expression of Bcl-2 in SH-SY5Y cells but not IMR-32 cells. In response to fenretinide treatment, Bcl-2 expression was down-regulated in both proliferating and ATRA pre-treated IMR-32 cells, but not in SH-SY5Y cells. Bcl-xL expression was also reduced in response to fenretinide in ATRA-treated compared to proliferating IMR-32 cells; conversely, there was evidence for slightly increased expression of Mcl-1 expression in IMR-32 cells in response to fenretinide. The ability of fenretinide to induce Noxa was maintained after ATRA treatment in both SH-SY5Y and IMR-32 cells, whereas the induction of the ER stress marker GADD153 was inhibited in ATRA-pre-treated SH-SY5Y cells. These data suggest that differential regulation of Bcl-2 after ATRA treatment may be responsible for the differences between SH-SY5Y and IMR-32 cells in fenretinide sensitivity.

Effect of RA on fenretinide metabolism. Experiments designed to investigate the influence of ATRA pre-treatment on fenretinide metabolism were carried out in SH-SY5Y cells, as this cell line most clearly exhibited an inhibitory effect of ATRA pre-treatment on fenretinide-induced apoptosis. Incubation of these cells with fenretinide did not lead to the generation of detectable levels of oxidative fenretinide metabo-

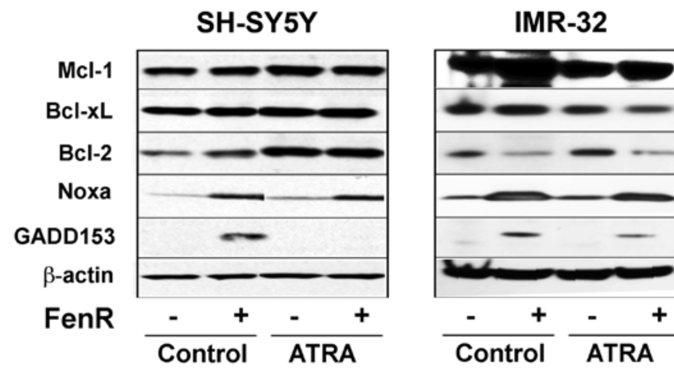

Figure 3. Impact of RA on Bcl-2 protein expression. SH-SY5Y and IMR-32 cells were pre-treated with ATRA (1 μM) for 5 days prior to washout and treatment with fenretinide (5 μM) for a further 24 h. Western blotting was performed for Mcl-1, Bcl-xL, Bcl-2, Noxa, GADD153 and β-actin.

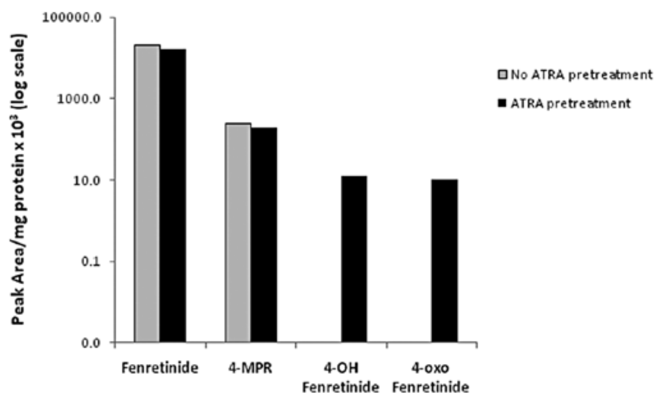

Figure 4. Intracellular levels of fenretinide and its metabolites 4-MPR, 4-OH-fenretinide and 4-oxo-fenretinide in SH-SY5Y cells. Cells were incubated with fenretinide (20 μM) for a period of 6 h following a 24-h pre-treatment with ATRA (10 μM) or no pre-treatment as shown. Metabolite peak area is expressed normalised to protein concentration and plotted on a log scale due to the large differences in levels between fenretinide and its metabolites (results are mean values, n=3; error bars not shown but <15% in all cases).

lites. Conversely, pre-treatment of SH-SY5Y neuroblastoma cells with ATRA, prior to incubation with fenretinide, resulted in the generation of 4'-OH-fenretinide and 4'-oxo-fenretinide metabolites. The production of these metabolites corresponded with an approximate 7-fold induction of CYP26A1 as compared to SH-SY5Y cells without RA preincubation. However, intracellular levels of the 4'-OH-fenretinide and 4'-oxo-fenretinide metabolites accounted for <10% of intracellular parent drug levels, with fenretinide levels not markedly reduced following ATRA pre-treatment (Fig. 4). Fenretinide levels in cells pre-treated with ATRA showed only a 7% decrease compared to levels observed in cells not pre-treated with ATRA. There was no effect of ATRA pre-treatment on intracellular concentrations of the other major fenretinide metabolite, 4-MPR.

Discussion

This study shows that, for a panel of neuronal (N)-type neuroblastoma cells with different genetic defects, differentiation with ATRA substantially reduced their sensitivity to fenretinide. 13cisRA is believed to work at a molecular level through isomerisation to ATRA in target neuroblastoma cells and in this study, both isomers had comparable properties with respect to reducing sensitivity to fenretinide. While ATRA treatment resulted in growth inhibition in all neuroblastoma cell lines used in this study, IMR-32 cells are generally regarded as RA-resistant, and exhibit a limited response in terms of cellular invasion, migration and expression of retinoid-regulated genes, particularly the retinoic acid receptors (RARs), after RA treatment (17). We demonstrate that the cell lines able to differentiate in response to RA treatment became resistant to fenretinide, whereas RA-resistant IMR-32 cells remained fenretinide sensitive. Focussed studies on SH-SY5Y and IMR-32 cells showed that resistance to fenretinide was accompanied by increased expression of Bcl-2, consistent with previous reports demonstrating regulation of Bcl-2 levels during neuroblastoma cell differentiation (7,18). An important element of the ER stress response, GADD153 activation, was abrogated in ATRA treated cells. However, the ER-stress-induced pathway of pro-apoptotic signalling in response to fenretinide appeared intact, as evidenced by Noxa induction. The down-regulation of Bcl-2 expression in IMR-32 cells in response to fenretinide, and the failure of RA treatment to increase Bcl-2 expression, is likely to be responsible for their increased apoptotic sensitivity compared to SH-SY5Y cells.

Experiments designed to investigate the influence of ATRA pre-treatment on fenretinide metabolism were carried out in cells which clearly exhibited an inhibitory effect of ATRA pre-treatment on fenretinide-induced apoptosis. Even at the relatively high ATRA and fenretinide concentrations utilised in these experiments, designed to optimise CYP26 induction and allow the detection of relatively low levels of fenretinide oxidative metabolites, it would appear that induction of fenretinide metabolism is unlikely to be a major factor influencing the apoptotic response to fenretinide after RA treatment. Although pre-treatment of SH-SH5Y cells with ATRA led to the generation of 4'-OH-fenretinide and 4'-oxo-fenretinide metabolites, presumably related to the observed induction of CYP26 expression, this increase in metabolism had very little effect on intracellular levels of fenretinide itself.

The use of different retinoid drugs in the treatment of neuroblastoma may have the potential to improve current patient survival rates. Treatment with 13cisRA for high-risk neuroblastoma takes place after high dose myeloablative

therapy and radiotherapy but the most appropriate way to utilise fenretinide remains unclear. *In vitro* data showing that neuroblastoma cells selected for 13cisRA resistance are sensitive to fenretinide suggest that fenretinide may be useful for MRD remaining after 13cisRA treatment (5). However, the mechanisms of action of 13cisRA *in vivo* are unknown and *in vitro* selection may not be an appropriate model of treatment failure since it has not been demonstrated that patients who relapse after retinoid therapy do so because of inherent retinoid resistance. If fenretinide is to be used after 13cisRA therapy for MRD then potential interactions with 13cisRA need be considered since, on the basis of these results, RA-differentiated cells are likely have increased expression of Bcl-2 and increased resistance to apoptotic agents such as fenretinide (6-8,18). This may be less important if 13cisRA-induced differentiation *in vivo* is reversible, but this is a question that has not currently been adequately addressed.

The findings from the current study could have important implications for the future use of retinoid drugs in neuroblastoma treatment, with careful consideration needed regarding the scheduling of fenretinide in clinical trials. Additional studies in preclinical animal models alongside further *in vitro* studies to more clearly define the mechanism of action of individual retinoids in neuroblastoma are warranted.

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