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Influence of isomerisation on the growth inhibitory effects and cellular activity of 13-*cis* and all-*trans* retinoic acid in neuroblastoma cells

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Abstract

Treatment with 13-*cis* retinoic acid (13-*cis* RA) has been shown to significantly improve the clinical outcome of children with high-risk neuroblastoma. Despite the large number of studies investigating the cellular effects of retinoids in neuroblastoma cells, the influence of RA isomerisation and the factors that determine the extent of RA isomerisation and uptake are unknown. The aim of this study was to establish the extent of extra- and intracellular isomerisation of 13-*cis* RA and all-*trans* retinoic acid (ATRA) in neuroblastoma cell lines, and to investigate the influence of isomerisation on their growth inhibitory effects and on the regulation of expression of cellular retinoic acid binding protein II (CRABP II) and RAR-β. Limited extracellular isomerisation was observed up to 72 hr after incubation of four neuroblastoma cell lines with 10 μM 13-*cis* RA or ATRA. The retinoic acid isomer present initially in the medium accounted for >75% of extracellular retinoid exposure. By contrast, incubation with 13-*cis* RA resulted in intracellular levels of ATRA comparable to those of 13-*cis* RA. This degree of intracellular isomerisation was not observed after ATRA incubations, with 13-*cis* RA accounting for <10% of total intracellular retinoids. No differences were observed in the sensitivity of three N-type neuroblastoma cell lines to either 13-*cis* RA (IC_{50}: 11.2–13.9 μM) or ATRA (IC_{50}: 12.9–14.4 μM), despite 10-fold differences in intracellular retinoid levels. A decrease in sensitivity to 13-*cis* RA (IC_{50} = 137 μM), as compared to ATRA (IC_{50} = 41 μM), was observed in the S-type cell line SH S EP. RAR-β was induced in a dose-dependent manner in SH SY 5Y cells following incubation with ATRA, whereas a weaker and delayed induction was observed with 13-*cis* RA. Similarly, incubation with ATRA resulted in a greater induction of CRABP II in these cells. In summary, these results indicate either an intracellular conversion of 13-*cis* RA to ATRA or a selective uptake of ATRA and suggest that this may mediate the differential activity of 13-*cis* RA in neuroblastoma cell subtypes.

Keywords: Retinoic acid; Neuroblastoma; Isomerisation; Cellular pharmacokinetics; Cytotoxicity; RAR-β; CRABP II

1. Introduction

Retinoids are a class of compounds consisting of both natural and synthetic substances structurally related to Vitamin A (retinol). They can inhibit cell proliferation and induce differentiation and apoptosis in many cell types during normal development as well as in cancer cells propagated in tissue culture [1–3]. Two of the most clinically useful retinol derivatives are 13-*cis* RA and ATRA. Despite the fact that these two retinoids are isomers, they exhibit contrasting efficacy, toxicity and pharmacokinetics in clinical studies [4,5]. Both 13-*cis* RA and ATRA have been used in clinical trials for the treatment of neuroblastoma and 13-*cis* RA has recently been shown to significantly improve the 3 year event-free survival of children with high-risk neuroblastoma when given for a 6 month period following myeloablative therapy [6].

Both 13-*cis* RA and ATRA have two major metabolic pathways, *cis–trans* isomerisation and oxidation to 4-oxoretinoic acid and 4-hydroxyretinoic acid [7]; these retinoid metabolites are also thought to play a role in determining the cellular effects of retinoids [8,9].

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Abbreviations: 13-*cis* RA, 13-*cis* retinoic acid; ATRA, all-*trans* retinoic acid; RAR, retinoic acid receptors; RXR, retinoid X receptors; CRBP, cellular retinol-binding protein; CRABP, cellular retinoic acid-binding protein; DMSO, dimethyl sulphoxide; PBS, phosphate buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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Retinoids are believed to exert most of their effects by binding to specific nuclear receptors, thus, modulating gene expression. The retinoid-response pathways are mediated by two subtypes of nuclear receptors: RAR (α, β and γ) and RXR (α, β and γ) [10,11]. The retinoic acid (RA) isomers differ in their affinities for the two types of receptor. 9-*cis* RA is a bifunctional ligand which can bind to both types of receptors whereas ATRA only transactivates RAR receptors. 13-*cis* RA does not bind to either receptor subtype with high affinity [12,13]. Despite a number of similarities between these two groups of retinoid receptors, it is thought that they have distinct roles in retinoid signalling [14]. Specific cellular retinoid-binding proteins, CRBP and CRABP, have also been identified and are involved in regulating the availability of the retinoids to nuclear receptors [15].

Many studies have described the effects of the various retinoids on neuroblastoma cell lines *in vitro*. ATRA induces apoptosis in S-type (substrate adherent) cells whereas differentiation occurs in N-type (neuroblastic) cells accompanied by induction of RAR-β. 9-*cis* RA is able to induce differentiation and apoptosis in both N- and S-type cells, probably producing its apoptotic effects via RXR [16].

As the RA isomers act through different receptors and elicit different cellular effects, retinoid responses are dependent on both the level of expression of specific receptor isotypes as well as the type and concentrations of individual isomers in the cell. It is clearly of critical importance to know the precise concentrations and nature of the retinoids present, as well as what retinoid receptors are expressed, when interpreting retinoid effects on cell growth and differentiation. However, despite the large number of published studies investigating the cellular effects of retinoids, the extent of RA isomerisation and its potential influence on the results obtained from these studies are largely unknown.

The current study was designed to determine the extent of extra- and intracellular isomerisation of 13-*cis* RA and ATRA in a panel of neuroblastoma cell lines commonly used to study cellular responses to retinoids. To investigate the influence of isomerisation on the *in vitro* effects of 13-*cis* RA and ATRA, the sensitivity of these cell lines to growth inhibition and the regulation of CRABP II and RAR-β expression was assessed in parallel with isomerisation studies.

2. Materials and methods

2.1. Cell lines

SH SY 5Y, SH S EP (N- and S-type cells, respectively, derived from the parental cell line SK N SH), IMR-32 and NGP neuroblastoma cell lines were cultured routinely in RPMI 1640 medium containing foetal calf serum (10%), L-glutamine (2 mM), penicillin (100 U/mL) and streptomycin (100 μg/mL). Cells were grown at 37° in a humidified atmosphere containing 5% CO_2 and were subcultured every 3 days.

2.2. Chemicals

13-*cis* RA, ATRA and 9-*cis* RA were purchased from Sigma. Both 13-*cis*-4-oxo RA (Ro 22-6595) and acitretin (Ro 10-1670) were generously provided by Hoffmann-La Roche. HPLC grade *n*-hexane, glacial acetic acid and 2-propanol and all other chemicals were obtained from Fisher Scientific.

2.3. Treatment with retinoids

Both 13-*cis* RA and ATRA were initially dissolved in DMSO and diluted in cell culture medium to obtain final concentrations of 0.01–1000 μM for cell growth inhibition studies and 10 μM for experiments investigating intra- and extracellular retinoid isomerisation. The final concentration of DMSO in the cell culture medium never exceeded 0.2% and an equivalent concentration of DMSO was added to control cells. All experiments were performed in dim light and tubes containing retinoids were wrapped in aluminium foil.

For growth inhibition studies, cells were seeded in 96-well plates at a concentration between 5000 and 10,000 cells per well in cell culture medium and were allowed to attach overnight. Either 13-*cis* RA or ATRA were added to a final concentration of 0.01–1000 μM. Growth was determined at 72 hr using the SRB assay to determine cellular protein as previously described [17,18]. Briefly, cells were fixed with trichloroacetic acid (TCA) and stained with 0.4% sulphorhodamine B in 1% acetic acid for 30 min. Unbound dye was removed by washing 5 times with 1% acetic acid and protein-bound dye was extracted with 10 mM unbuffered Tris base. Spectrophotometric measurement of the absorption at 540 nm was carried out using a 96-well plate reader. The IC_{50} values were calculated from absorption values and defined as the concentration corresponding to a 50% growth reduction compared with values for untreated control cells.

For studies investigating the extent of retinoid isomerisation, neuroblastoma cells were seeded at various cell densities and allowed to attach overnight. Cell culture medium containing 13-*cis* RA or ATRA was added to give a final concentration of 10 μM and cells were grown in the presence of retinoids for up to 72 hr. Medium was taken at 0, 6, 24, 48 and 72 hr for extracellular retinoid measurements. Cells were isolated at 6, 24, 48 and 72 hr by addition of trypsin (0.05%)/EDTA (0.02%), washed twice with PBS and the cell number determined for intracellular retinoid measurements. All samples were stored at −20° in foil wrapped tubes prior to extraction of retinoids and analysis by HPLC.

To investigate the effect of retinoic acid isomers on the expression of RAR-β and CRABP II, SH SY 5Y and SH S EP neuroblastoma cells were seeded in tissue culture flasks and allowed to attach overnight. Cell culture medium containing 13-*cis* RA or ATRA was added to give final concentrations ranging from 0.01 to 10 μM and cells were grown in the presence of retinoids for 6 and 24 hr prior to RNA extraction and Northern blotting.

2.4. Extraction of retinoids

Cell pellets obtained from retinoid incubations were resuspended in 2 mL cell culture medium and disrupted by passing the sample several times through a syringe and hypodermic needle (25G × 1 in., 0.5 mm × 25 mm; Terumo). Proteins were precipitated by adding 500 μL of ethanol containing the internal standard acitretin and 500 μL of saturated ammonium sulphate solution to 2 mL of extracellular medium or resuspended cells. Samples were vortex mixed and an extraction mixture containing *n*-hexane, dichloromethane and propan-2-ol (80:19:1) added prior to extraction on a rotary mixer for 10 min. After centrifugation at 2000 *g* for 30 min at 4°, the organic layer was removed and evaporated under a stream of nitrogen. Samples were reconstituted in 200 μL *n*-hexane and a 100 μL aliquot analysed by HPLC. All procedures were carried out in dim light using aluminium foil-wrapped tubes.

2.5. HPLC analysis

Quantification of intra- and extracellular retinoid levels was carried out by HPLC analysis using a Waters 2690 Separations Module and 996 Photodiode array (PDA) detector (Waters Ltd.) and Waters Millennium software for data acquisition.

Separation was performed on a silica gel column (Nucleosil 100, 5 μm, 25 cm × 4.6 mm) fitted with a silica gel precolumn (Nucleosil 100, 5 μm, 3 cm × 4.6 mm) using a method modified from that previously reported by Lanvers *et al.* [19]. Analysis was performed at a constant flow rate of 2.5 mL/min and a run time of 40 min per sample. The gradient ran between 0 and 15 min with mobile phase A (*n*-hexane:dichloromethane:propan-2-ol, 400:1:0.27) and changed linearly to mobile phase B (*n*-hexane:dichloromethane:propan-2-ol, 400:6:0.27) between 15 and 16 min. Mobile phase B ran from 16 to 30 min and changed linearly back to A between 30 and 31 min. Mobile phase A then ran until 40 min. Quantification of retinoids was performed by comparison of peak areas at 350 nm using authentic standards (0.02, 0.05, 0.10, 0.2, 0.5, 1, 2, 5 and 10 μg/ml) of 13-*cis* RA, ATRA, 9-*cis* RA and 13-*cis*-4-oxo RA extracted by liquid–liquid extraction as described above.

Cell volumes were determined as previously described [18] and RA concentrations converted from μg/mL to μM based on these results in conjunction with cell counts and the molecular weight of retinoic acid.

2.6. RNA extraction and Northern blotting

Cells were detached from the culture flasks with 1 mL of 0.05% (w/v) trypsin/0.02% (w/v) EDTA. Trypsin was neutralised with 0.25 mL of FBS and the cells washed once with 0.5 mL of PBS. Cells were resuspended in 0.4 mL of PBS and cytoplasmic RNA extracted by lysis with Nonidet P40 and sodium deoxycholate as described by Wilkinson [20]. Nuclei were removed by centrifugation at 6000 *g* for 30 s and the supernatant extracted once with 500 μL of 4:1 phenol:chloroform, 1% SDS, twice each with 500 μL of 1:1 phenol:chloroform and once with 500 μL of chloroform. After ethanol precipitation, RNA samples (20 μg per track) were size-fractionated by electrophoresis through 1.2% agarose/formaldehyde gels, transferred to nylon membranes (Amersham International) and probed with ^{32}P-labelled cDNA probes for human RAR-β [21], human CRABP II [22] and rat GAPDH [23] as described previously [24]. Blots were exposed to phosphor storage screens, scanned in a Molecular Dynamics phosphorimager and band intensities quantified using ImageMaster (Amersham Pharmacia) software. Band intensities for RAR-β and CRABP II were corrected for loading using the GAPDH signal intensity and expressed relative to control cells treated with vehicle (DMSO) only.

2.7. Statistical analysis

Statistical significance of the data was evaluated by the two-sided Student's *t*-test with a probability level of 0.05 indicating significant differences.

3. Results

3.1. Analysis of extracellular retinoid concentrations

SH SY 5Y, SH S EP, IMR-32 and NGP neuroblastoma cell lines were treated with 13-*cis* RA or ATRA (10 μM) for up to 72 hr and extracellular concentrations in cell culture medium determined by HPLC analysis. These data show the actual exposures of these cell lines to individual retinoic acid isomers following incubations with 13-*cis* RA and ATRA. However, it should be stated that low levels of isomerisation were observed following incubations of retinoids with cell culture medium in the absence of cells as previously reported by others [25].

After addition of 13-*cis* RA, extracellular concentrations of 13-*cis* RA decreased with incubation time in all cell lines (Fig. 1A). Isomerisation to ATRA and 9-*cis* RA over a 72 hr period was negligible with >80% of extracellular retinoid exposure accounted for by 13-*cis* RA, i.e. <20%

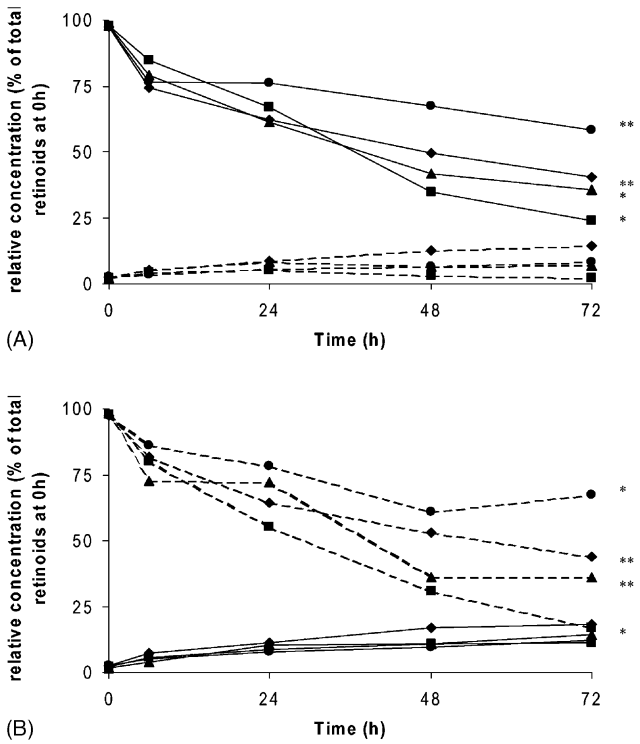

Fig. 1. Measurement of 13-*cis* RA (solid line) and ATRA (broken line) in cell culture medium following incubation of SH SY 5Y (■), SH S EP (●), IMR-32 (◆) and NGP (▲) neuroblastoma cell lines with 10 μM 13-*cis* RA (A) or ATRA (B). Values are expressed as a percentage of the total retinoids measured at 0 hr. Data are mean values from at least three separate experiments, standard deviations are omitted for clarity but were <20% in all cases. The statistical significance of the difference between exposures ($AUC^{ext}_{0-72\,hr}$) to 13-*cis* RA and ATRA are shown for each cell line. $^{*}P < 0.05$; $^{**}P < 0.01$.

conversion to ATRA and 9-*cis* RA, as determined from area under the extracellular medium concentration–time curves ($AUC^{ext}_{0-72\,hr}$) for each isomer (Table 1). The rates at which 13-*cis* RA disappeared from the extracellular medium was reflected in the 13-*cis* RA $AUC^{ext}_{0-72\,hr}$ values, and ranged from 185 μM hr (mean value from three separate experiments) in NGP cells to 343 μM hr in SH S EP cells. A similar pattern was seen after incubations with ATRA (Fig. 1B), with extracellular ATRA concentrations decreasing with incubation time. Isomerisation to 13-*cis* RA and 9-*cis* RA was limited with >75% of extracellular retinoid exposure accounted for by ATRA (Table 1). Exposures to ATRA were generally higher than for 13-*cis* RA with ATRA $AUC^{ext}_{0-72\,hr}$ values ranging from 287 μM hr in SH SY 5Y cells to 386 μM hr in SH S EP cells. Apart from the retinoic acid isomers, no metabolites of 13-*cis* RA or ATRA were detected in extracellular medium at any of the time points studied during the 72 hr incubations.

3.2. Analysis of intracellular retinoid concentrations

Intracellular retinoid concentrations were quantified in SH SY 5Y, SH S EP, IMR-32 and NGP cells at 6, 24, 48 and 72 hr after incubation with 10 μM 13-*cis* RA or ATRA. Results are expressed as mean ± SD from at least three separate experiments.

Intracellular 13-*cis* RA concentrations determined after incubation with 13-*cis* RA were up to 10-fold higher than extracellular concentrations. Peak levels were seen at 6 or 24 hr in all cell lines and ranged from 8.4 ± 1.7 μM in SH SY 5Y cells to 72.1 ± 22.8 μM in SH S EP cells (Fig. 2). Intracellular concentrations of ATRA, after addition of 13-*cis* RA to the culture medium, accounted for up to 42% of total intracellular retinoids as determined from individual retinoid area under the intracellular concentration–time curves ($AUC^{int}_{0-72\,hr}$; Table 2). Intracellular ATRA concentrations were higher than 13-*cis* RA concentrations in IMR-32 cells at 48 hr (35.8 ± 3.9 μM vs. 30.7 ± 6.1 μM) and 72 hr (34.2 ± 17.5 μM vs. 23.3 ± 16.9 μM) and in

Table 1
Extracellular exposures to individual retinoic acid isomers following incubation with 10 μM 13-*cis* RA or ATRA[a]

Cell line	RA isomer	13-*cis* RA incubation		ATRA incubation	
		$AUC^{ext}_{0-72\,hr}$ (μM hr)	Percentage of total retinoids	$AUC^{ext}_{0-72\,hr}$ (μM hr)	Percentage of total retinoids
SH SY 5Y	13-*cis* RA	194 ± 58.7	89	57.3 ± 26.2	15
	9-*cis* RA	9.6 ± 3.5	5	25.6 ± 15.4	7
	ATRA	13.7 ± 1.8	6	287 ± 39.3	78
SH S EP	13-*cis* RA	343 ± 35.1	91	44.8 ± 7.2	10
	9-*cis* RA	7.5 ± 1.1	2	12.2 ± 0.8	3
	ATRA	27.7 ± 6.9	7	386 ± 81.2	87
IMR-32	13-*cis* RA	284 ± 46.4	81	71.9 ± 4.5	17
	9-*cis* RA	18.3 ± 2.7	5	27.1 ± 2.3	6
	ATRA	46.8 ± 5.0	14	328 ± 58.7	77
NGP	13-*cis* RA	185 ± 79.4	83	59.7 ± 7.9	14
	9-*cis* RA	14.7 ± 0.8	7	31.3 ± 10.0	7
	ATRA	22.1 ± 7.3	10	338 ± 65.3	79

[a] Results are expressed as mean ± SD from $n \geq 3$ experiments. $AUC^{ext}_{0-72\,hr}$ represents the retinoid area under the extracellular medium concentration–time curve calculated between 0 and 72 hr using the trapezoidal rule.

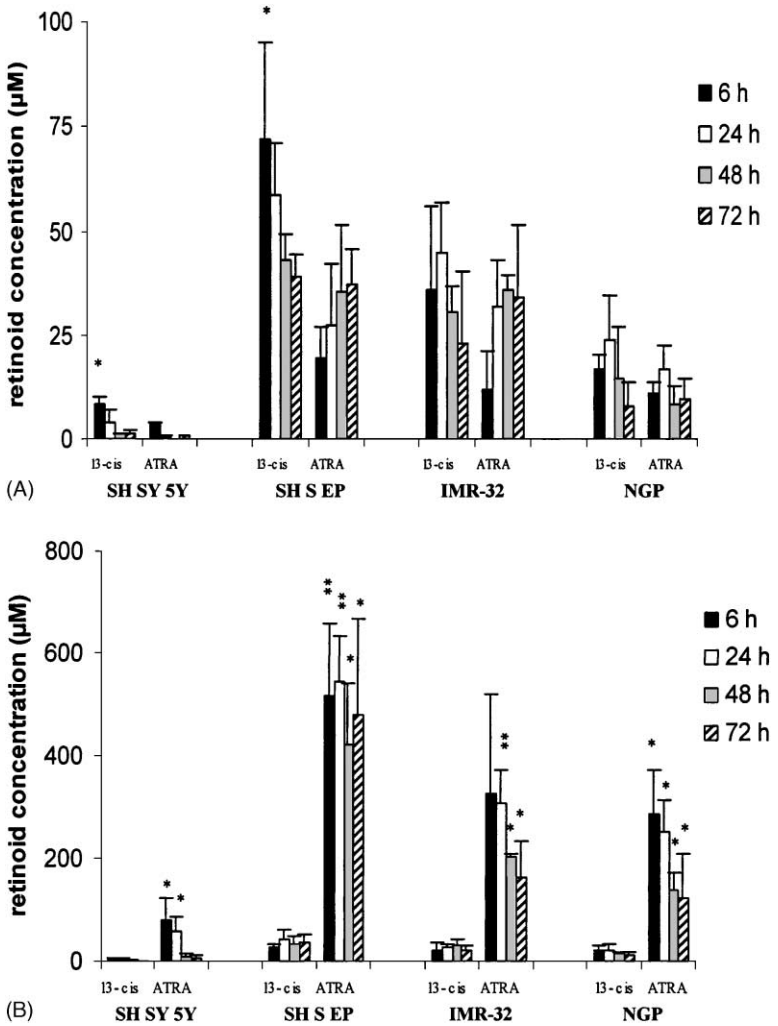

Fig. 2. Cellular retinoic acid disposition in neuroblastoma cell lines at various time points up to 72 hr following incubation with 10 μM 13-*cis* RA (A) or ATRA (B). Values are expressed as intracellular concentration of retinoic acid based on cell counts and cell volume calculation. Data are mean values ± SD from at least three separate experiments. The statistical significance of the difference between concentrations of 13-*cis* RA and ATRA at individual time points for each cell line are shown. $^{*}P < 0.05$; $^{**}P < 0.01$.

Table 2
Intracellular exposures to individual retinoic acid isomers following incubation with 10 μM 13-*cis* RA or ATRA[a]

Cell line	RA isomer	13-*cis* RA incubation		ATRA incubation	
		$AUC^{int}_{0-72\,hr}$ (μM hr)	Percentage of total retinoids	$AUC^{int}_{0-72\,hr}$ (μM hr)	Percentage of total retinoids
SH SY 5Y	13-*cis* RA	337 ± 46.2	78	168 ± 20.9	5
	9-*cis* RA	ND	–	48.6 ± 3.1	2
	ATRA	97.3 ± 5.1	22	3045 ± 204	93
SH S EP	13-*cis* RA	3584 ± 309	67	2066 ± 575	6
	9-*cis* RA	136 ± 83.7	2	403 ± 44.0	1
	ATRA	1637 ± 64.8	31	32442 ± 6476	93
IMR-32	13-*cis* RA	2736 ± 848	55	1759 ± 110	9
	9-*cis* RA	146 ± 57.0	3	327 ± 113	2
	ATRA	2119 ± 605	42	18312 ± 4843	89
NGP	13-*cis* RA	1151 ± 555	56	1236 ± 259	8
	9-*cis* RA	98.8 ± 4.1	5	304 ± 82.3	2
	ATRA	811 ± 233	39	13497 ± 2805	90

[a] Results are expressed as mean ± SD from $n \geq 3$ experiments. $AUC^{int}_{0-72\,hr}$ represents the retinoid area under the intracellular medium concentration–time curve calculated between 0 and 72 hr using the trapezoidal rule. ND—not determined.

NGP cells at 72 hr (10.0 ± 4.5 μM vs. 8.0 ± 5.7 μM). In SH SY 5Y and SH S EP cells, intracellular concentrations of ATRA were equivalent to 13-*cis* RA concentrations at 72 hr. No significant difference in intracellular concentrations of 13-*cis* RA and ATRA were observed between 24 and 72 hr, following incubation with 13-*cis* RA, in any of the cell lines.

Incubations of neuroblastoma cells with ATRA resulted in intracellular concentrations of ATRA approximately 10–100-fold higher than extracellular concentrations. Peak ATRA levels were up to 12-fold higher than intracellular concentrations of 13-*cis* RA after incubation of cells with 13-*cis* RA under similar conditions. Peak intracellular ATRA concentrations were seen between 6 and 24 hr and ranged from 80.0 ± 42.6 μM in SH SY 5Y cells to 544 ± 88.2 μM in SH S EP cells (Fig. 2). Limited intracellular isomerisation to 13-*cis* RA was observed after ATRA incubations and intracellular concentrations of 13-*cis* RA accounted for <10% of total intracellular retinoids as determined from individual retinoid $AUC^{int}_{0-72\,hr}$ (Table 2). Intracellular concentrations of 9-*cis* RA accounted for ≤5% of total retinoids after incubations with either 13-*cis* RA or ATRA in all cell lines.

3.3. Effects of retinoids on cell proliferation

The SRB assay was used to assess the antiproliferative effect of 13-*cis* RA and ATRA in SH SY 5Y, SH S EP, IMR-32 and NGP neuroblastoma cells. The IC_{50} values for individual cell lines were calculated from growth inhibition curves from three separate experiments (Fig. 3). Comparable IC_{50} values were determined for 13-*cis* RA in SH SY 5Y, IMR-32 and NGP cells (14.4, 13.3 and 12.9 μM, respectively) with a 10-fold higher IC_{50} value of 137 μM observed for SH S EP cells. A similar pattern was seen after incubations with ATRA with IC_{50} values of 13.4, 13.9 and 11.2 μM in SH SY 5Y, IMR-32 and NGP cells respectively and an IC_{50} value of 40.6 μM determined for the less sensitive SH S EP cells.

3.4. Effects of retinoids on RAR-β and CRABP II expression

Expression of RAR-β and CRABP II was determined in SH SY 5Y (N-type) and SH S EP (S-type) cells at 6 and 24 hr following treatment with 13-*cis* RA or ATRA (0.01–10 μM). Fig. 4 shows induction of RAR-β and CRABP II expression in SH SY 5Y cells after a 24 hr incubation with 13-*cis* RA and ATRA.

RAR-β was induced in a dose-dependent manner in SH SY 5Y cells after incubation with ATRA (Fig. 5). An approximate 5-fold increase in RAR-β expression relative to the control was observed at 6 hr with an ATRA concentration of 10 μM. Incubation with 13-*cis* RA resulted in a weaker and delayed induction of RAR-β, with a maximum 2.5-fold increase in expression at 24 hr after incubation with 10 μM 13-*cis* RA (Table 3). No induction of RAR-β expression was observed in the S-type SH S EP cells in response to treatment with either 13-*cis* RA or ATRA.

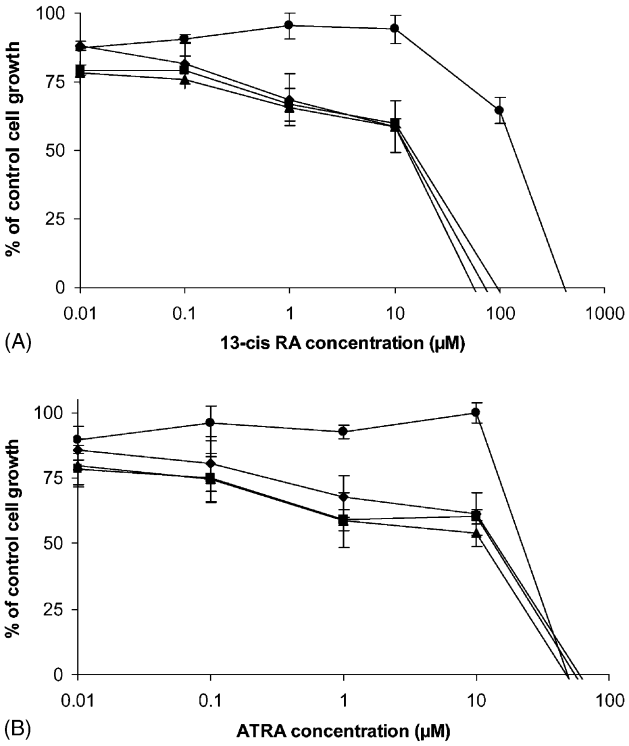

Fig. 3. Measurement of the antiproliferative effect of 13-*cis* RA (A) and ATRA (B) in SH SY 5Y (■), SH S EP (●), IMR-32 (◆) and NGP (▲) neuroblastoma cell lines using the SRB assay. Values are expressed as a percentage of the control cell growth determined in cell culture medium in the absence of retinoids. Data are mean values ± SD from three separate experiments.

CRABP II expression was induced in a dose-dependent manner in SH SY 5Y cells treated with both ATRA and 13-*cis* RA (Fig. 5). Incubation with 10 μM ATRA for 6 hr resulted in a 7-fold increase in CRABP II expression

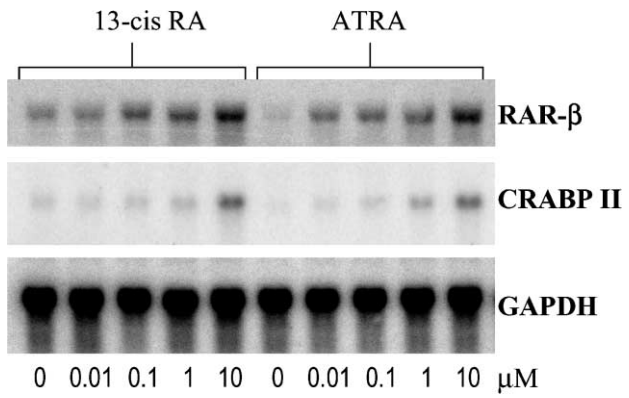

Fig. 4. Induction of expression of RAR-β and CRABP II determined by Northern blot analysis in SH SY 5Y cells after 24 hr incubations with 13-*cis* RA or ATRA (0.01–10 μM). GAPDH was used as a reference to correct for RNA loading.

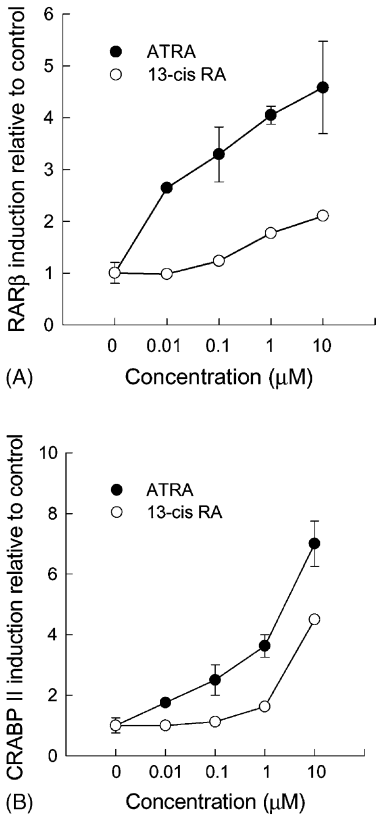

Fig. 5. Effect of retinoic acid concentration on induction of RAR-β (A) and CRABP II (B) following incubations of SH SY 5Y cells with 13-*cis* RA (○) or ATRA (●) for 6 hr. Values are expressed as a proportion of the untreated control and corrected for variations in RNA loading using the GAPDH signal. Data are mean values with error bars showing the range from two experiments.

relative to the control as compared to a 4.5-fold increase after a comparable 13-*cis* RA treatment (Table 3). A greater induction of CRABP II expression was observed at 24 hr, with approximately 15- and 11-fold increases relative to the control after incubation with 10 μM ATRA and 13-*cis* RA, respectively. No expression of CRABP II was seen in SH S EP cells in either control or retinoid treated cells.

Table 3
Effect of incubation time on the induction of expression of RAR-β and CRABP II following incubation of SH SY 5Y cells with 10 μM 13-*cis* RA or ATRA[a]

	RA isomer	Induction of expression relative to control (hr)	
		6	24
RAR-β	13-*cis* RA	2.1	2.5
	ATRA	4.6	3.8
CRABP II	13-*cis* RA	4.5	11.4
	ATRA	7.0	14.7

[a] Values are expressed as a proportion of the untreated control and corrected for variations in RNA loading using the GAPDH signal. Results are mean values from $n = 2$ experiments.

4. Discussion

It has recently been demonstrated that despite the standard precautions taken when working with retinoids in the laboratory, the presence of thiol-containing compounds in cell culture medium may result in significant RA isomerisation [25]. This may influence the intracellular concentrations of specific RA isomers after incubation of cell lines with retinoids *in vitro*. The current study was designed to determine the extent of extra- and intracellular isomerisation of 13-*cis* RA and ATRA in a panel of neuroblastoma cell lines. To establish the influence of isomerisation on the *in vitro* effects of 13-*cis* RA and ATRA, the sensitivity of these cell lines to inhibition of proliferation and induction of gene expression was assessed in parallel with isomerisation studies.

In this study, limited extracellular isomerisation was observed up to 72 hr after addition of either 13-*cis* RA or ATRA to neuroblastoma cell lines. Isomerisation to 9-*cis* RA and ATRA accounted for <20% of extracellular retinoid exposure over this time period after addition of 13-*cis* RA. A similar pattern was seen after ATRA treatment, with isomerisation to 9-*cis* RA and 13-*cis* RA accounting for <25% of extracellular retinoid exposure. It is interesting to note that the disappearance of the parent RA, whether 13-*cis* RA or ATRA, was slower in incubations with SH S EP neuroblastoma cells. These cells also exhibited a reduced sensitivity to 13-*cis* RA and ATRA. It has previously been reported that the extent of growth inhibition induced by ATRA in head and neck squamous cell carcinoma cell lines was proportional to the rate at which ATRA, 13-*cis* RA and 9-*cis* RA disappeared from the cell culture medium [26].

Extracellular retinoid concentrations did not correlate with intracellular levels of retinoic acids. Intracellular retinoic acid concentrations after incubation with 13-*cis* RA varied substantially between cell lines, with total intracellular retinoid exposures for SH S EP cells being approximately 15-fold greater than in SH SY 5Y cells. In contrast, the variation in extracellular retinoid exposures was less than 2-fold. Incubation of neuroblastoma cell lines with 13-*cis* RA resulted in similar intracellular exposures to both 13-*cis* RA and ATRA, with ATRA concentrations actually exceeding those of 13-*cis* RA in IMR-32 and NGP cells at later time points. As there was only very limited extracellular isomerisation of 13-*cis* RA in cell culture medium, these data suggest either selective intracellular isomerisation of 13-*cis* RA to ATRA or an increased cellular uptake of ATRA in neuroblastoma cells.

After incubation with ATRA, intracellular retinoid concentrations were approximately 10–100-fold higher than extracellular concentrations. Intracellular ATRA concentrations were approximately 10-fold higher than those of either 13-*cis* RA or ATRA after 13-*cis* RA incubation and intracellular 13-*cis* RA levels were comparable to those observed after 13-*cis* RA incubation. As extracellular

retinoid exposures to these two retinoic isomers following their respective incubations were comparable, with limited extracellular isomerisation observed, these results strongly suggest a selective uptake of ATRA into neuroblastoma cells.

With respect to growth inhibition, comparable IC_{50} values were determined for both 13-*cis* RA and ATRA in SH SY 5Y, IMR-32 and NGP neuroblastoma cells. The most striking observation from these experiments was the lack of correlation between intracellular retinoid levels and the growth inhibitory effects of the retinoids on these cell lines. Despite a 10-fold increase in intracellular retinoid levels after incubation with ATRA, as compared to incubations with 13-*cis* RA, comparable IC_{50} values were observed for 13-*cis* RA and ATRA in these cell lines. Our data also allows us to look at possible correlations between individual retinoic acid isomer intracellular exposures and growth inhibitory effects. Intracellular levels of 13-*cis* RA were more closely related to IC_{50} values than those of ATRA, with very similar intracellular exposures to 13-*cis* RA following incubation with either retinoic acid isomer reflected in the comparable IC_{50} values observed. However, the most likely reason for the lack of correlation between intracellular retinoid concentrations and the growth inhibitory effects in these cell lines, is a saturation of retinoid receptors at these high retinoic acid concentrations. ATRA has previously been reported to bind with high affinity to RAR receptors with K_d estimates ranging from 0.2 to 0.4 nM [13,27].

The S-type SH S EP cells were less sensitive to the retinoids than the other cell lines studied, with estimated IC_{50} values approximately 10 and 3-fold higher for 13-*cis* RA and ATRA, respectively. This may be related to a different mechanism of action of the retinoids in this cell line, as ATRA is thought to predominantly induce apoptosis, as opposed to cellular differentiation, in S-type neuroblastoma cells [28,29]. If this is the case, the higher IC_{50} value observed in these cells for 13-*cis* RA may be explained by lower intracellular ATRA levels following incubation with 13-*cis* RA; intracellular 13-*cis* RA concentrations were comparable after incubation with either retinoic acid isomer.

The regulation of expression of CRABP II and RAR-β were used as cellular markers of retinoid activity in SH SY 5Y and SH S EP cells. These cell lines represent N- and S-type cell lines, respectively, from the same parental SK N SH cell line, showed the lowest (SH SY 5Y) and highest (SH S EP) intracellular concentrations of 13-*cis* RA and ATRA and exhibited a difference in cytotoxicity between 13-*cis* RA and ATRA in our experiments. RAR-β was induced in a dose-dependent manner in SH SY 5Y cells in response to incubation with ATRA, with a weaker and slower induction obtained with 13-*cis* RA over the same concentration range. This decreased and delayed rate of RAR-β induction by 13-*cis* RA may be due to isomerisation of 13-*cis* RA to ATRA and subsequent induction of RAR-β by ATRA. This possibility is supported by results from experiments investigating CRABP II expression, with 13-*cis* RA again exhibiting a reduced induction of expression as compared to ATRA. CRABP II is thought to regulate free intracellular retinoic acid incubations by promoting metabolism of ATRA, and its expression has been shown to be induced by ATRA itself [15]. No induction of RAR-β expression was observed in SH S EP cells over the concentration range studied and CRABP II expression could not be detected in this cell line. As CRABP II is involved in the regulation of intracellular retinoid levels, its lack of expression in SH S EP cells might explain the higher intracellular retinoid concentrations observed in this cell line following incubations with both ATRA and 13-*cis* RA. The lack of induction of RAR-β in SH S EP cells may indicate a different mechanism of action of the retinoids in this S-type cell line. This could explain the decreased sensitivity of these cells, despite the high intracellular retinoid levels observed after treatment with 13-*cis* RA and ATRA.

This study highlights a number of important issues concerning the extent of retinoic acid isomerisation and its possible influence on the growth inhibitory and cellular activity of retinoic acids in neuroblastoma cells. Results from *in vitro* experiments carried out to investigate the effects of 13-*cis* RA on neuroblastoma cells may well reflect the extent of 13-*cis* RA isomerisation and selective uptake of ATRA into these cells. This will have a significant impact on the conclusions drawn from studies with single retinoic acid isomers. As 13-*cis* RA now plays a key role in the treatment of children with neuroblastoma, it is essential that both *in vitro* and *in vivo* studies continue to allow the optimisation of its use in a clinical setting. The influence of retinoic acid isomerisation on the results obtained from clinical studies should be carefully considered.

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