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## Original Paper

# Retinoid-induced Differentiation of Neuroblastoma: Comparison between LG69, an RXR-selective Analogue and 9-*cis* Retinoic Acid

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**The aim of this study was to investigate *in vitro* the effects of all-*trans* retinoic acid (RA), 9-*cis* RA and the RXR-selective analogue, LG69, on the morphological differentiation, proliferation and gene expression of neuroblastoma cells. Three different cell lines were cultured with the retinoid for either 9 continuous days or for 5 days followed by 4 days without the retinoid and morphological differentiation was assessed both qualitatively and quantitatively. SH SY 5Y cell proliferation was examined by measuring cell numbers after exposure to the retinoids and RAR-β gene expression was examined by Northern blot analysis. Morphological differentiation was more effectively induced by all-*trans* and 9-*cis* RA than by LG69. SH SY 5Y cells, when treated with 9-*cis* RA for only 5 of the 9 days of culture, underwent apoptosis, but this was not seen with 9 days continuous exposure nor with LG69. Inhibition of SH SY 5Y cell proliferation by all-*trans* or 9-*cis* RA was dose-dependent, but LG69 had little effect. Conversely, LG69 induced higher expression of RAR-β than all-*trans* RA, but less than that produced by 9-*cis* RA. These data suggest that 9-*cis* RA as a single agent is the most effective modulator of neuroblastoma behaviour and may be the most appropriate therapeutic agent.** © **1998 Elsevier Science Ltd.**

**Key words: neuroblastoma, retinoid, 9-*cis* retinoic acid, differentiation, gene expression, proliferation, apoptosis**
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## INTRODUCTION

NEUROBLASTOMA IS thought to arise from neural crest cells and several clinical features suggest that a disordered process of differentiation may be involved, at least, in part, in its pathogenesis. Spontaneous regression, via an apoptotic differentiation pathway [1], is reported to occur more often than in any other tumour [2] and maturation to differentiated ganglioneuroma, both spontaneously and with treatment, is well documented [3–5]. This maturation, however, is not a universal phenomenon and emphasises that neuroblastoma is a heterogeneous disease, in terms of both clinical behaviour and the phenotypic diversity of tumour cells. Despite intensive multimodality treatment, patients with metastatic disease still have an extremely grave prognosis. As differentiation can be induced both *in vivo* and *in vitro*, neuroblastoma represents an ideal tumour for a more innovative approach to therapy, particularly in patients with poor prognostic disease. Consequently, both all-*trans* and 13-*cis* retinoic acid (RA) have been used clinically, on the basis of their *in vitro* differentiating effects [6]. While results from two large multicentre randomised trials of 13-*cis* RA in neuroblastoma patients are currently awaited, published results to date, in terms of sustained remissions, have been disappointing [7–9].

RA is thought to mediate its effects via nuclear receptors, RA receptors (RARs) and retinoid 'X' receptors (RXRs), which are expressed in neuroblastoma cells [10, 11]. RA induces the expression of RAR-β in several neuroblastoma

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cell lines [10, 12] and this receptor may also have prognostic significance *in vivo* [11], as it does in head and neck cancer [13, 14]. All-*trans* RA is the ligand for RARs and 9-*cis* RA binds with high affinity to both RARs and RXRs [15]. Since 13-*cis* RA only binds weakly to nuclear RA receptors (RARs), *in vivo* responses to 13-*cis* RA may result from isomerisation to all-*trans* RA. 9-*cis* RA is more effective at inducing RAR-β in neuroblastoma cells than all-*trans* or 13-*cis* RA [16–18] and, as for acute promyelocytic leukaemia cells [19–21] and breast cancer cells [22–24], is more effective at inducing differentiation *in vitro* [17, 25–27]. In terms of inhibition of proliferation of neuroblastoma cells, 9-*cis* RA is as effective and in some reports more effective than all-*trans* RA [17, 18, 25–27]. As a result of these *in vitro* findings, 9-*cis* RA may be a more appropriate retinoid for neuroblastoma therapy.

One of the potential limitations for the clinical use of 9-*cis* RA, is its propensity to isomerise to less effective isomers. Side-effects of the RA isomers, perhaps as a consequence of their ability to activate multiple retinoid receptors in a variety of target tissues, will also be dose limiting [28, 29]. Consequently, the development of novel, receptor-selective retinoids with greater stability *in vivo* and which mimic the biological properties of 9-*cis* RA may have important therapeutic implications for neuroblastoma treatment. We have previously suggested that the greater effects of 9-*cis* RA may be mediated via the activation of RXR homodimers [16, 25, 30]. A number of RXR-selective compounds have been identified [31, 32]. Of these, LGD1069, 4-[1-(3,5,5,8,8-pentamethyl-5,6,7,8-terahydro-2-naphthyl) ethenyl] benzoic acid (compound **6b** of Boehm and associates [31] and referred to here as LG69), is the first RXR-selective retinoid to enter clinical trials for cancer and is currently being evaluated in the treatment of non-small cell lung cancer, renal cell carcinoma (in combination with interferon α) and Kaposi's sarcoma [31]. LG69 may thus be an alternative to 9-*cis* RA for neuroblastoma therapy and the purpose of this study was to determine whether or not LG69 has similar biological properties to 9-*cis* RA. Using the SH SY 5Y cell line as a model of RA-induced differentiation, we investigated the differential effects of all-*trans* or 9-*cis* RA and the RXR-selective analogue LG69, on morphological differentiation, proliferation and gene expression of neuroblastoma. In addition, we examined whether the biologically distinct effects of 9-*cis* RA on SH SY 5Y cells are common to other neuroblastoma cell lines.

## MATERIALS AND METHODS

*Morphological differentiation*

$2\times10^3$ cells/ml were seeded into 25 $cm^2$ tissue culture flasks, in triplicate and incubated overnight in Dulbecco's modification of Eagle's medium containing 2 mM L-glutamine, supplemented with 10% fetal calf serum (culture medium) at 37°C in a humidified atmosphere of 5% $CO_2$ in air. All-*trans* RA, 9-*cis* RA or LG69 were added to a final concentration of $10^{-6}$ M. The RA isomers were added in ethanol and LG69 was added in DMSO. An equal volume of ethanol or DMSO was added to control plates. The culture medium was changed every 2 days and replaced with fresh medium containing the appropriate retinoid, analogue or control reagent. At day 5, half the flasks were rinsed with PBS, thereby removing the retinoid, analogue, DMSO or ethanol, replacing it with medium alone and in the other half, the medium was replaced with fresh medium plus the retinoid, analogue or control reagent and cells were cultured for a further 4 days with fresh medium changes (containing the appropriate reagent) every second day. The cells were photographed after 3, 5, 7 and 9 days. Neurites were measured with Vernier callipers from photomicrographs and the median neurite length/cell determined and plotted as a quantitative measure of differentiation [25]. Confidence limits were calculated using the Minitab (Minitab Inc., State College, Pennsylvania, U.S.A.) statistical software.

*Proliferation assay*

$0.75\times10^3$ cells were plated into 96-well-flat-bottom tissue culture plates in 200 µl of culture medium and incubated overnight before adding all-*trans* RA, 9-*cis* RA (in concentrations from $10^{-9}$ to $10^{-6}$ M) or LG69 ($10^{-9}$ to $10^{-5}$ M). An equal volume of ethanol or DMSO was added to control cells. The culture medium was replaced every second day with fresh medium containing the RA isomer, analogue, ethanol or DMSO. The cell number after 5 days was assessed colorimetrically after fixing and staining the cells with crystal violet [33]. The optical density was measured at 594 nm, using an MC 340 Titertek multiscan plate reader.

*Gene expression*

The expression of RAR-β and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, RNA loading control) was examined by Northern blotting. $8\times10^6$ SH SY 5Y cells were seeded into 75 $cm^2$ flasks ($1.07\times10^5$ cells/$cm^2$ growth area) and incubated in culture medium at 37°C with 5% $CO_2$ in air. After overnight attachment, $10^{-10}$ to $10^{-6}$ M all-*trans* or 9-*cis* RA, or the RXR-selective analogue, LG69 ($10^{-9}$ to $10^{-5}$ M) were added. RA was added in an ethanol solution and an equal volume of ethanol was added to control flasks. LG69 was added in DMSO and control flasks containing equal volumes of DMSO were also prepared. After 6 h incubation with reagents, total cytoplasmic RNA was extracted using the 'miniprep' method [34], size-fractionated by electrophoresis through 1.2% agarose/formaldehyde gels and transferred by vacuum blotting to nylon membranes [35]. Membranes were then probed consecutively with $^{32}$P-labelled cDNA probes for RAR-β and GAPDH [36] and autoradiographed.

## RESULTS

*Morphological differentiation*

All-*trans* RA is a powerful inducer of morphological differentiation in neuroblastoma cells [6], but recent investigations have shown that 9-*cis* RA is the most effective isomer at promoting differentiation [17, 25, 26] and also has distinct biological effects, inducing apoptosis after treatment and subsequent washout [37]. The relative morphological effects of LG69, 9-*cis* RA, all-*trans* RA, ethanol or DMSO after either 9 days of continuous treatment with retinoids at $10^{-6}$ M or treatment with retinoids for 5 days followed by 4 days culture in the absence of retinoid are shown in Figure 1. The extension of processes from the cell body (neurites) are indicators of neuronal differentiation [38] and were more pronounced in the all-*trans* or 9-*cis* RA treated cells than in the LG69 or control cells. Cell density was greatest in the control and LG69-treated cultures, suggesting that LG69 did not appreciably inhibit proliferation. After removal of the agents, there were some striking morphological differences. The appearance of cells that had been pre-treated with 9-*cis* RA had features indicative of apoptosis: i.e. smaller, contracted

cells, with loss of cell–cell interconnection and neurites [39]. Conversely, in the LG69 and all-*trans* RA pre-treated cultures, there was a return to the original undifferentiated state with the cells regaining their proliferative capacity (Figure 1). This was not as evident in the 9-*cis* RA pre-treated cells.

The marked effects of 9-*cis* RA on SH SY 5Y cells after treatment and subsequent washout were not confined to this neuroblastoma cell line. SK N SH cells, a mixed cell line of N- and S-type cells from which SH SY 5Y cells were originally derived [40], also responded to 9-*cis* RA treatment and washout in a similar manner to SH SY 5Y cells (Figure 2a, c and e). However, in this cell line, it is not clear if the morphologically distinct cells remaining in the cultures were residual S-type cells, or if differentiated N-type cells reverted to an S-phenotype before initiating apoptosis. The N-type GI LI N cells grew as flat colonies of cells, mostly lacking neurites in the absence of RA, but in the continuous presence of 9-*cis* RA neurite outgrowth was stimulated. After treatment with 9-*cis* RA for 5 days and subsequent washout, cell density was markedly reduced, colonies of cells became denser and clumped, with detachment and extensive cell death (Figure 2b, d and f).

To assess morphological changes more objectively, the numbers and lengths of neurites per cell were measured and the median total length/cell plotted against time. The relative neurite lengths of cells with continuous treatment or after washout at 5 days are shown in Figure 3. In agreement with previous data [25], 9-*cis* RA was quantitatively more effective at inducing neurite extension than any of the other agents. In both continuous and washout conditions, 9-*cis* RA induced an increase in neurite length until day 5; this was, to some extent, sustained under continuous treatment with 9-*cis* RA (Figure 3b), but declined to control values within 2 days of washout (Figure 3a). LG69 was no more effective than control treatments at inducing neurite extension.

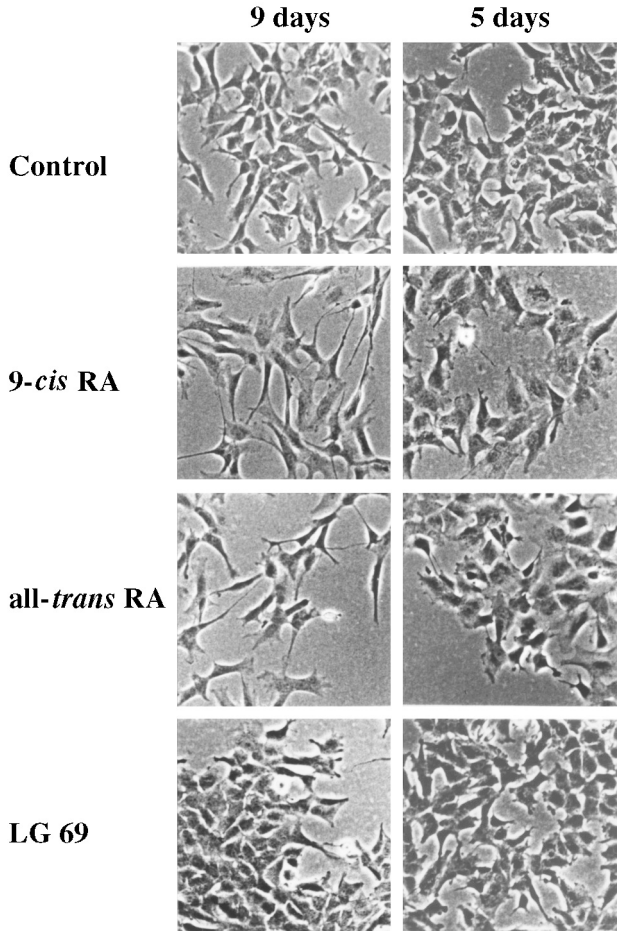


**Figure 1. Morphological differentiation of SH SY 5Y cells in response to retinoids. Cells were treated with 1μM 9-*cis* RA, all-*trans* RA or the RXR-selective analogue LG69 for 9 days continuously or for 5 days, followed by washout and culture in the absence of retinoid for a further 4 days. (Magnification ×400.)**

*Inhibition of proliferation*

RA is also an effective inhibitor of proliferation of SH SY 5Y cells [25, 26, 41], reducing cell number either by increasing cell cycle time, or promoting increased cell death (apoptosis) or growth arrest. As LG69 was not as effective as either all-*trans* or 9-*cis* RA at inducing differentiation in these cells, we compared its anti-proliferative activity with all-*trans* and 9-*cis* RA. Both 9-*cis* and all-*trans* produced dose-dependent inhibition of SH SY 5Y cell proliferation: the effects of all-*trans* and 9-*cis* RA were similar for concentrations greater than $10^{-8}$ M, with all-*trans* RA more effective than 9-*cis* RA at lower concentrations (Figure 4). LG69 was ineffective at inhibiting proliferation, producing a significant decrease in cell number only at the highest doses used ($10^{-6}$ and $10^{-5}$ M), but even at these concentrations it was considerably less effective than all-*trans* or 9-*cis* RA.

*Gene expression: induction of RAR-β by 9-*cis *RA and LG69*

9-*cis* RA has been shown to be a more effective inducer of RAR-β than all-*trans* RA in a dose-dependent manner in SH SY 5Y cells [16]. To determine whether the RXR-selective analogue was effective at gene induction, RAR-β expression was examined after treatment of the cells for 6 h with either 9-*cis* RA ($10^{-10}$ to $10^{-6}$ M), all-*trans* RA ($10^{-10}$ to $10^{-6}$ M) or LG69 ($10^{-9}$ to $10^{-5}$ M). As for 9-*cis* RA, LG69 induced RAR-β in a dose-dependent manner. Although the peak induction achieved with LG69 was less than that with 9-*cis* RA, this was greater than with all-*trans* RA (Figure 5). As LG69 is RXR-selective, this suggests that 9-*cis* RA may be inducing RAR-β via RXRs. These data support previous reports of the greater efficacy of 9-*cis* RA to induce RAR-β [16, 17, 27].

## DISCUSSION

Retinoids have demonstrated potential in the prevention and treatment of a number of malignancies and their actions are thought to be mediated through nuclear receptors. There is increasing evidence that the mechanism of action of RA varies according to which isomer predominates. All-*trans* RA binds to RARs but not to RXRs and 9-*cis* RA binds with similar affinity to RARs and RXRs [15]. All-*trans* RA must therefore exert its effects via RARs while 9-*cis* RA may have the potential to exert its activity via both RARs and RXRs. As the RXR-selective analogue LG69 induces RAR-β, this implies that, in SH SY 5Y cells, 9-*cis* RA may induce gene expression, in the short term, principally via RXRs, perhaps as RXR-homodimers [16, 26]. Experiments with receptor-selective antagonists have supported the idea that 9-*cis* and all-*trans* RA do indeed work by different receptor mechanisms [41]. RXRs also act as auxiliary proteins or dimer partners for thyroid hormone, vitamin D3 and peroxisome

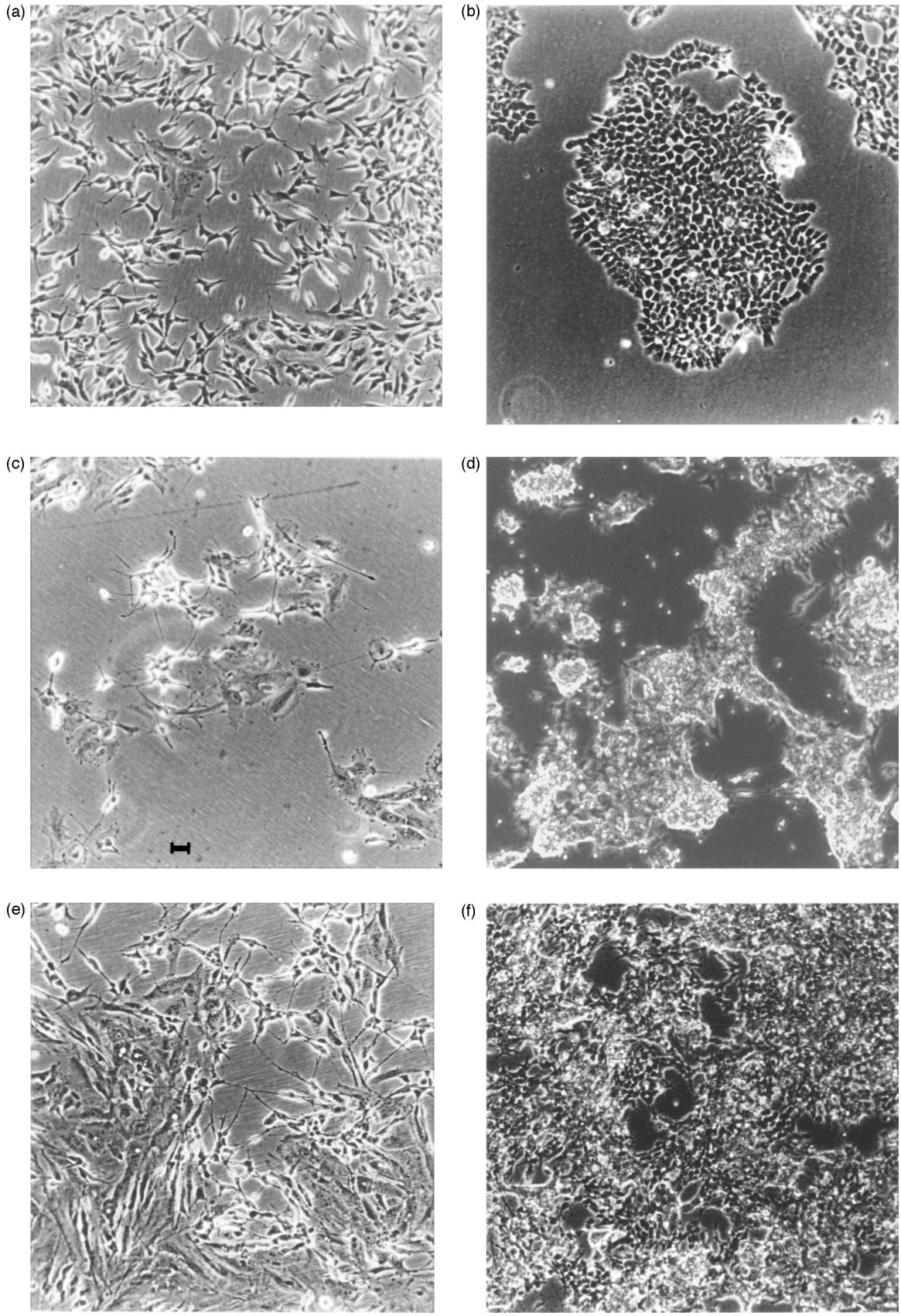


**Figure 2. Morphological differentiation of SK N SH and GI LI N neuroblastoma cells in response to 9-*cis* RA. SK N SH (a, c, e) and GI LI N cells (b, d, f) were treated with 9-*cis* RA continuously for 9 days (e) and (f), or for 5 days followed by washout and further culture for 4 days in the absence of 9-*cis* RA (c and d). Cells cultured with ethanol as the vehicle control are shown in (a) and (b). Magnification on all phase-contrast photomicrographs is ×400; bar on (c) is 10 μM.**

proliferator receptors [42]. Elucidating the precise mechanism of action of 9-*cis* RA is further complicated by recent findings that 9-*cis* RA can activate the RXR partner in heterodimers between other nuclear receptors such as LXRs [43], and the 'orphan receptors' NURR-1 and NGFI-B [44]. These latter two, closely related receptors are encoded by immediate-early genes, activated by a variety of cell stimulatory factors, and this has implications for the role of retinoids and RXRs in cell growth and differentiation [44]. Although 9-*cis* RA is very effective at inducing morphological differentiation *in vitro*, toxicity *in vivo* may be a factor limiting its use in children. In clinical trials of 9-*cis* RA in adults, the dose limiting toxicities were headache and diarrhoea [28, 29]. Hypercalcaemia has also been reported and it is possible that this may be more pronounced in children, as is the case with 13-*cis* RA [45]. In view of these limitations, RXR-selective analogues such as LG69 may be important alternatives for clinical use if efficacy and lack of side-effects can be demonstrated.

The results from this study confirm the greater effect of 9-*cis* RA on the morphological differentiation of SH SY 5Y neuroblastoma cells, compared with other RA isomers and analogues and further suggest that the ability of 9-*cis* RA to induce apoptosis after treatment and subsequent washout is not uniquely limited to SH SY 5Y cells, but occurs in other

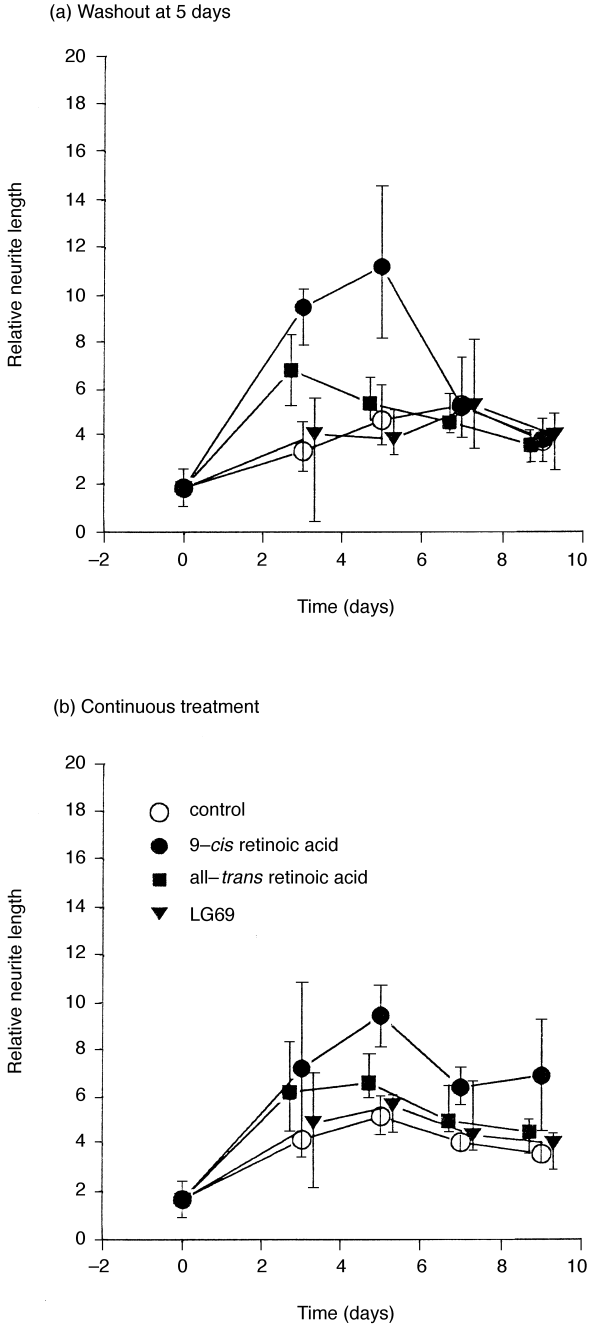


**Figure 3. Differentiation of SH SY 5Y cells in response to retinoids. Relative neurite lengths (total length per cell) at 0, 3, 5, 7 and 9 days after treatment with 1 μM 9-*cis* RA, all-*trans* RA or the RXR-selective analogue LG69 for 9 days continuously (b) or for 5 days, followed by washout and culture in the absence of retinoid for a further 4 days (a). Each point represents a median, ±95% confidence interval calculated using the Minitab SINTERVAL program.**

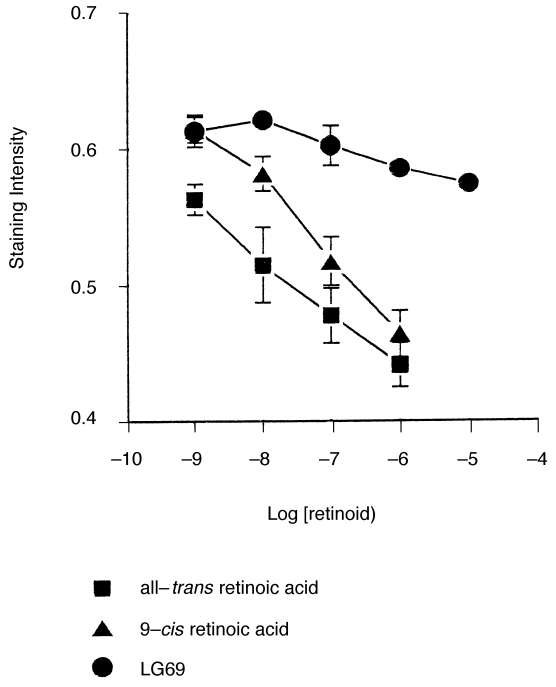


**Figure 4. Inhibition of SH SY 5Y cell proliferation after culture for 5 days with all-*trans* RA, 9-*cis* RA or LG69. Staining intensity (optical density at 594 nm), a measure of cell number, is shown plotted against $\log_{10}$ retinoid concentration. Error bars are ±95% confidence limits, $n = 12$ per treatment.**

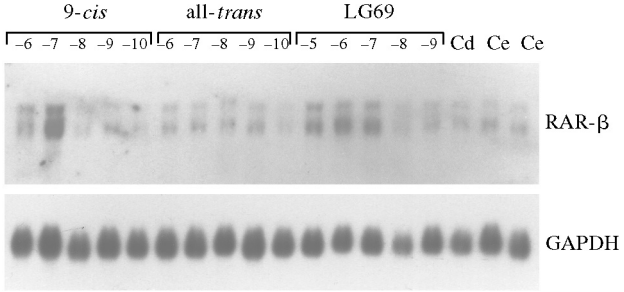


**Figure 5. Dose-dependent induction of RAR-β in response to 9-*cis* RA, all-*trans* RA or the RXR-selective agonist LG69. Northern blot of RNA extracted from SH SY 5Y cells treated for 6 h with either $10^{-10}$ to $10^{-7}$ M 9-*cis* or all-*trans* RA or $10^{-9}$ to $10^{-6}$ M LG69, and probed successively for RAR-β (full length probe) and GAPDH. Ce, control ethanol; Cd, control DMSO.**

neuroblastoma cell lines as well. Unlike 9-*cis* and all-*trans* RA and despite its ability to induce RAR-β expression, the RXR-selective analogue LG69 was relatively ineffective at inducing morphological differentiation and inhibiting proliferation. Previous work has shown that 9-*cis* RA is better than all-*trans* RA at inducing gene expression in short-term induction experiments, inducing morphological differentiation and inhibiting the proliferation of SH SY 5Y neuroblastoma cells [16, 25–27]. Since LG69 is effective at inducing RAR-β, but relatively ineffective at inducing morphological differentiation and inhibiting proliferation, these events can be dissociated. Thus, these data indicate that short-term induction of RAR-β is mediated by a different receptor mechanism to morphological differentiation and inhibition of proliferation. Furthermore, the apparent dissociation between RAR-β induction and morphological differentiation suggests that RAR-β expression is insufficient to induce morphological differentiation and raises the question of whether the induction of RAR-β has any role in facilitating differentiation in response to retinoids.

The fact that LG69 was ineffective at inducing differentiation implies that RXR activation alone is insufficient and that RAR activation may be required to promote differentiation, with co-activation of RXRs necessary for a full differentiation response. This model would explain the limited differentiation response obtained with all-*trans* RA, a result of RAR-RXR heterodimer activation via the RAR partner alone. Differentiation in response to 9-*cis* RA may be due either to co-activation of RAR-RXR heterodimers with all-*trans* RA formed by isomerisation or via binding of 9-*cis* to both RAR and RXR partners, or to a combined response of 9-*cis* RA-dependent RXR homodimers and RAR-RXR heterodimers. This implies that the apoptosis of SH SY 5Y cells after 9-*cis* RA treatment and washout [37] may be driven by RXR activation as a consequence of RAR-dependent morphological differentiation.

Although 9-*cis* RA is, on balance, more effective than all-*trans* RA at inhibiting proliferation, at least at high ($10^{-6}$ M) concentrations, the difference in activity between these two isomers is not marked by comparison with the differentiation response. The mechanism of proliferation inhibition in response to RA is not known, although two, not mutually exclusive, mechanisms have been outlined. All-*trans* RA has been shown to cause a decrease in the expression of $p34^{cdc2}$ cyclin-dependent kinase in neuroblastoma cells with a consequent block of cells in the G1 phase of the cell cycle [46]. Decreased expression of this or other cell-cycle control proteins in response to all-*trans* or 9-*cis* RA is thus one mechanism which would result in reduced proliferation. The AP1 transcription factor plays an important part in promoting cell growth and it has been shown that nuclear receptors such as RARs and RXRs can bind as ligand-dependent receptor monomers to the *fos* and *jun* components of AP1, thus reducing AP1 activity [47]. If this mechanism operates in SH SY 5Y cells, the relative effects of the two isomers would depend on the relative abundance of RAR and RXR monomers. The ability of ligands to activate receptor dimers on response elements and to promote receptor–AP1 interactions seems to depend on different functional groups on the ligand and anti-proliferative retinoid analogues, capable of promoting receptor-AP1 interactions but not receptor-dimer activation, have been synthesised [48]. LG69 may thus lack the necessary functional groups to promote receptor-AP1 interactions and this may be an explanation for its lack of anti-proliferative activity.

This study indicates that 9-*cis* RA is the most effective retinoid for inducing neuroblastoma differentiation *in vitro*. However, further work is required to show that 9-*cis* RA is also effective in *in vivo* models. Nevertheless, 9-*cis* RA has more-favourable pharmacokinetic properties *in vivo* than other retinoic acid isomers [30] and this is an important factor supporting 9-*cis* RA as the most appropriate retinoid for clinical trials. The *in vitro* data for SH SY 5Y cells suggest that morphological differentiation of neuroblastoma cells may be dependent on RAR-activation, with apoptosis resulting from RXR-activation followed by retinoid washout. If this is indeed the pathway of 9-*cis*-induced differentiation, it is possible that a combination of an RAR-selective retinoid, such as all-*trans* RA or a synthetic RAR-selective analogue, with an RXR-selective retinoid such as LG69, may be as effective as 9-*cis* RA without the problems of RA toxicity. Further work is required to address this issue. In the meantime, the combined effects of morphological differentiation followed by apoptosis after retinoid washout, indicate that 9-*cis* RA could be a valuable adjunct to conventional chemotherapy for minimal residual metastatic disease or for treatment of the more-localised disease for which the current treatment is a short course of chemotherapy.


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