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Gene Expression and Neuroblastoma Cell Differentiation in Response to Retinoic Acid: Differential Effects of 9-*cis* and All-*trans* Retinoic Acid

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Retinoic acid has considerable potential for the chemoprevention and chemotherapy of cancer. Neuroblastoma cells differentiate in response to retinoic acid *in vitro*, an observation that has led to clinical trials using either the 13-*cis* or all-*trans* isomers of retinoic acid. We review the effects of retinoic acid on neuroblastoma, and the potential involvement of nuclear retinoic acid receptors (RARs) and retinoid X receptors (RXRs). 9-*cis* retinoic acid is a ligand for RXRs, and we review recent data on the differential effects of 9-*cis* and all-*trans* retinoic acid on neuroblastoma differentiation and proliferation *in vitro*, and possible mechanisms of action via hetero- and homodimers of RARs and RXRs. Although there is uncertainty whether or not 9-*cis* retinoic acid produces its biological effects primarily via RXR homodimers, *in vitro* data suggest that this isomer of retinoic acid or stable analogues may have considerable potential for the treatment of resistant, disseminated neuroblastoma.

Key words: retinoids, all-*trans* retinoic acid, 9-*cis* retinoic acid, RAR-β, CRABP II, gene expression, receptors, neuroblastoma

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INTRODUCTION

VITAMIN A has long been recognised as important for development and growth. The discovery and cloning of nuclear retinoic acid receptors [1–3] has led to increased interest in the role of this small molecule, a derivative of vitamin A, in controlling cell differentiation. Neuroblastoma cells, like teratocarcinoma and promyelocytic cells, undergo marked morphological changes *in vitro* when treated with retinoic acid [4], and this observation has resulted in clinical trials to establish whether or not retinoic acid is effective as a form of treatment for neuroblastoma *in vivo*. Preliminary studies, in which either the 13-*cis* or all-*trans* isomers of retinoic acid have been used [5, 6], suggest that retinoic acid may be of value in some patients, and fully randomised trials are now in progress. Recent advances in our understanding of the mode of action of retinoic acid at a molecular level, particularly with respect to its role as a transcriptional regulator, suggest that new generations of synthetic retinoids designed to mimic an hitherto relatively obscure retinoic acid isomer, 9-*cis* retinoic acid (Figure 1), may have considerable clinical potential.

DIFFERENTIATION OF SH-SY-5Y CELLS IN RESPONSE TO 9-*CIS* AND ALL-*TRANS* RETINOIC ACID

Phenotypic relationships within and between human neuroblastoma cell lines are complex, indicating that, clinically, neuroblastoma may not be a single disease. Neuroblast (N-type), substrate-adherent (S-type) and intermediate (I-type) cells have been recognised within well-established lines [7, 8]. I-type cells may be precursors to N- and S-type cells [7, 8], which may, nevertheless, retain the ability to transdifferentiate [9–11]. Retinoic acid induces the differentiation of N-, S- and I-type cells,

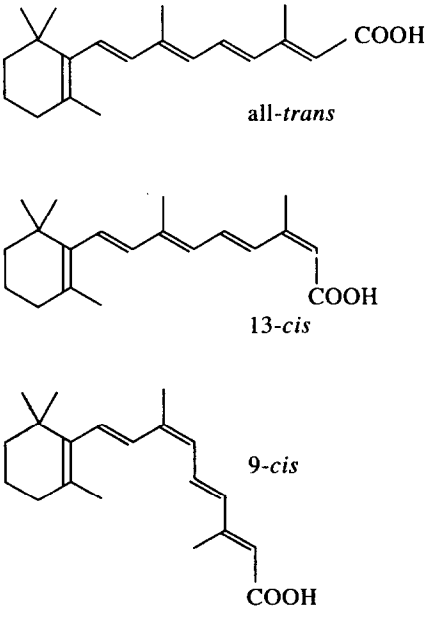

Figure 1. All-*trans*, 13-*cis* and 9-*cis* retinoic acid.

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but the resulting phenotype depends on the cell line and particular sub-clone: S-type cells may differentiate to Schwannian [9] or melanocytic types [10], or undergo apoptosis [12]. The SH-SY-5Y cell line is a neuroblast or N-type line derived from the SK-N-SH cells [13], and neuronal differentiation is induced by either retinoic acid [14] or dibutyryl cyclic AMP (dbcAMP) [15]; both these agents induce the extension of cell processes, here referred to as neurites, but biochemical phenotypes may differ [14]. Although most differentiation experiments with human neuroblastoma cells have been carried out with relatively high retinoic acid concentrations of 10^{-6}–10^{-5} M, in our hands neurite extension in SH-SY-5Y cells after 2 days exposure to all-*trans* retinoic acid reaches a maximum at 10^{-8} M, and does not increase with higher doses of retinoic acid or longer incubation times of up to 4 days [16] (Figure 2). Conversely, dbcAMP is more effective at promoting morphological differentiation, and neurite length continues to increase with time during 4 days incubation [16] (Figure 2).

In contrast to all-*trans* and 13-*cis*, 9-*cis* retinoic acid has a greater effect on cell morphology, with neurite length continuing to increase with time over at least 4 days *in vitro*, comparable to treatment with dbcAMP alone (Figure 2). Dose–response studies with all three retinoic acid isomers have demonstrated that 9-*cis* retinoic acid is less effective than the other isomers at low concentrations (10^{-8} M), but more effective at higher concentrations of 10^{-6} M [16] (Figure 3). At a concentration of 10^{-6} M, 13-*cis* retinoic acid produces a slightly greater response than an equivalent dose of all-*trans* [16]. Whether or not this is a result of isomerisation to all-*trans* requires further study to elucidate the extent of isomerisation under these culture conditions, the relative rates of metabolism of the different retinoic acid isomers, and, in consequence, the area under the concentration curve for all-*trans* retinoic acid after incubation with all-*trans* or 13-*cis* retinoic acid, respectively.

In addition to promoting morphological change, all-*trans* retinoic acid inhibits neuroblastoma cell proliferation, which may be due, at least in some cell lines, to reduced expression of cyclin-dependent protein kinases [17]. In SH-SY-5Y cells, reduced proliferation in response to all-*trans* retinoic acid is not a result of cytotoxicity [16], and is dose-dependent, giving a linear decrease in proliferation rate for all-*trans* retinoic acid concentrations ranging over at least 4 orders of magnitude

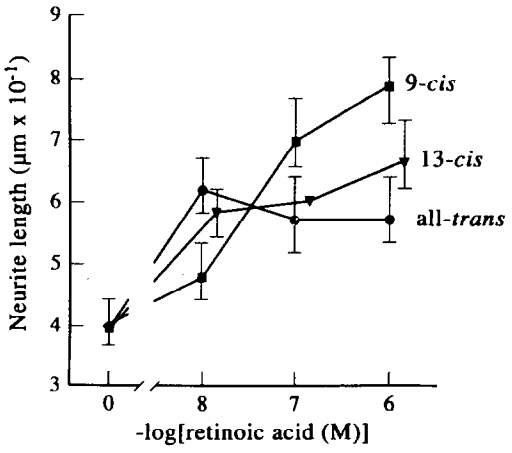

Figure 3. Dose–response studies of morphological differentiation induced by retinoic acid isomers in SH-SY-5Y cells after 2 days. Each point is the median ± 95% CI. For details see [16].

from 10^{-10} M [16] (Figure 4). 9-*cis* retinoic acid also inhibits proliferation to a similar extent to all-*trans* at 10^{-7}–10^{-6} M, but is less effective at lower doses (Figure 4).

THE MECHANISM OF ACTION OF RETINOIC ACID

In embryonal carcinoma (EC) cells, retinoic acid-induced differentiation is accompanied by changes in gene expression within a few hours of exposure to retinoic acid [18]. This may be a direct result of the binding of all-*trans* retinoic acid to nuclear retinoic acid receptors (RARs). The three types of RAR (RAR-α, -β and -γ) are ligand-dependent transcriptional regulators, closely related in structure to steroid and thyroid hormone receptors [2, 3, 19], and act by binding as receptor dimers to retinoic acid response elements (RAREs), specific DNA sequences linked in *cis* to retinoic acid-responsive genes. Although earlier studies of retinoic acid-induced transcription were indicative of transcriptional activation via RAR homodimers, it now appears that RARs have considerably greater activity as heterodimers with auxiliary proteins [20, 21]. These auxiliary proteins, called retinoid X receptors or RXRs, are also members of the steroid receptor superfamily and bind 9-*cis*

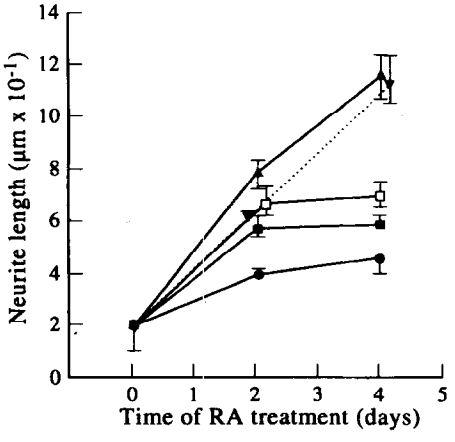

Figure 2. Morphological differentiation of SH-SY-5Y cells treated with retinoic acid (1 μM) isomers or dbcAMP [from 16]. Each point is the median ± 95% confidence interval (CI). ●, control; ■, all-*trans*; □, 13-*cis*; ▲, 9-*cis*; ▼, dbcAMP.

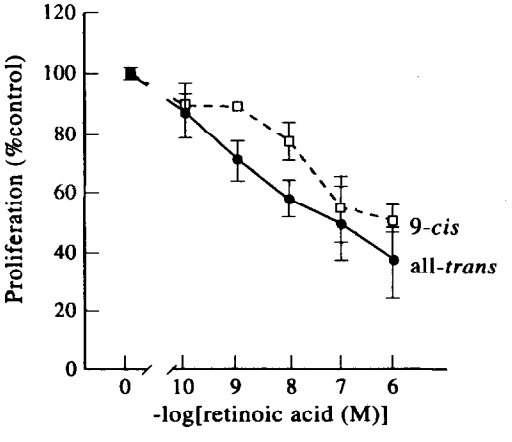

Figure 4. Logistic growth curve constants for SH-SY-5Y cells as a function of increasing all-*trans* or 9-*cis* retinoic acid concentration (from [16]). Data expressed as a percentage of the logistic growth rate constants for the control cells in each of three experiments. Error bars ± S.E.M.

retinoic acid with high affinity [22, 23]. Thus, RARs may work predominantly as heterodimers with RXRs [24], although the possibility that RAR homodimers also have a role *in vivo* cannot be excluded [25–27]. All-*trans* retinoic acid binds with high affinity (K_d 0.2–0.4 nM [28]) to RARs but not to RXRs. In contrast, 9-*cis* retinoic acid, a minor component in equilibrium mixtures of retinoic acid isomers [29, 30], binds with a comparable affinity to RXRs (K_d 1.4–2.4 nM) and also to RARs (K_d 0.2–0.8 nM) [28]. A property of RXRs, which is of particular importance, is that they act as auxiliary proteins or dimer partners for a number of other nuclear hormone receptors including thyroid hormone, vitamin D_3 and peroxisome proliferator receptors [24], and 9-*cis* retinoic acid is thus a *potentially* important ligand for co-regulating other hormone response pathways. Direct repeats (DR) of the sequence (A/G)GTTCA separated by 1 (DR + 1) to 5 (DR + 5) nucleotides form the basis of response elements for RAR–RXR heterodimers and for hetero- and homodimers for RXRs and other ligand-dependent transcriptional regulators [24].

The involvement of RXRs in retinoic acid-mediated transcriptional responses raises a number of questions with respect to the role of different retinoic acid isomers in neuroblastoma differentiation. For example, does 9-*cis* retinoic acid play a role in transcriptional activation resulting in neuroblastoma differentiation? Is RAR–RXR heterodimer activity independent of 9-*cis* retinoic acid, and can 9-*cis* act synergistically with all-*trans* retinoic acid (Figure 5)? Answers to these questions are important in order to make the most effective clinical use of retinoids in the treatment of neuroblastoma.

GENE EXPRESSION IN NEUROBLASTOMA CELLS IN RESPONSE TO 9-*CIS* AND ALL-*TRANS* RETINOIC ACID

The differentiation of human neuroblastoma cells by all-*trans* retinoic acid is accompanied by changes in gene expression. At the mRNA level, both induction and repression has been described, with the genes involved falling into two broad categories: those, such as *RAR-β* [31, 32], *RAR-α* [33], interleukin-8 [34], *IGF-II* [35], thrombospondin [36], the *RET* proto-oncogene [37] and *MYCN* [38] which are induced or repressed within a few hours, and those which change in expression after 2 or more days, such as *PGY1* [39], *GAP43* [40], βA4-amyloid [41], *POMC* [42] and *ID2* [43]. Furthermore, N- and S-type cells differ in their responses with, for example, mRNA for tissue transglutaminase showing rapid induction in S-type cells only [12], whereas the induction of the *RET* proto-oncogene is confined to N-type cells [37]. In SH-SY-5Y neuroblastoma cells, the expression of RAR-β is rapidly induced in response to all-*trans* retinoic acid [44] (Figure 6). Since the *RAR-β* gene has been relatively well characterised and its induction may be important for the retinoic acid-mediated expression of "late" genes, it may be a valuable marker for elucidating the mechanism of action of different retinoic acid isomers. For this reason, we have studied the induction of *RAR-β* in SH-SY-5Y cells in detail and compared its induction characteristics in response to retinoic acid isomers with another gene, *CRABP II*.

RAR-β mRNAs are transcribed from either of two promoters [45, 46], and the internal human RAR-β2 promoter contains a DR + 5 RARE (βRARE) [47]. RAR-β transcription is upregulated in response to retinoic acid in HepG2 cells [48], and this is also likely to be the mechanism of RAR-β mRNA induction in other cell types [49]. In SH-SY-5Y cells, RAR-β induction in response to all-*trans* retinoic acid is rapid (Figure 6), and

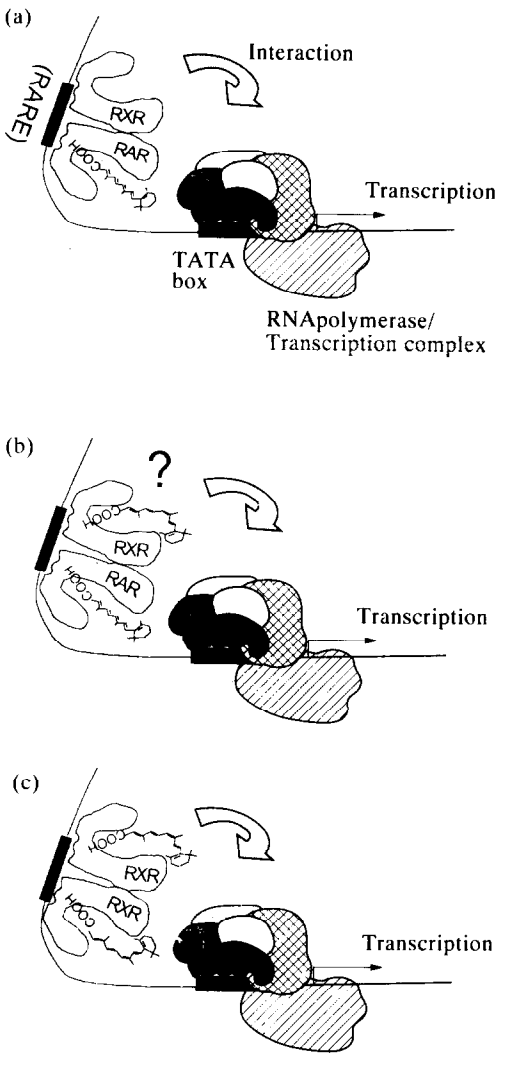

Figure 5. Potential modes of transcriptional regulation by retinoic acid receptors. In (a), the binding of all-*trans* retinoic acid to the RAR partner of the RXR–RAR heterodimer facilitates transcriptional activation via interactions with the transcription complex independently of RXR ligand. In (b), 9-*cis* retinoic acid may synergise with all-*trans*, giving an enhanced response to RXR–RAR heterodimers. In (c), 9-*cis* retinoic acid induces the formation of RXR homodimers [61] potentially allowing qualitatively and quantitatively different transcriptional activating properties to RXR–RAR heterodimers.

abolished by actinomycin D, but not inhibitors of protein synthesis [31, 44]. This evidence suggests that RAR-β transcription is induced by all-*trans* retinoic acid in SH-SY-5Y cells.

Messenger RNA levels for cellular retinoic acid binding protein II (CRABP II) also increase rapidly in SH-SY-5Y cells in response to all-*trans* retinoic acid [44] (Figure 6). *CRABP II* is transcriptionally regulated by retinoic acid in human fibroblasts [50] and murine embryonal carcinoma cells [51]. The murine *CRABP II* (*mCRABP II*) promoter has two RAREs [51], and at least one putative DR + 1-type RARE has been described within the human *CRABP II* promoter [50]. Since the induction of *CRABP II* by all-*trans* retinoic acid in SH-SY-5Y cells is rapid, abolished by actinomycin D and insensitive to inhibitors of protein synthesis [44], this is also likely to be directly mediated at a transcriptional level. Moreover, there are no apparent changes in CRABP II mRNA stability in response to retinoic acid [44]. Unlike RAR-β, where mRNA levels after induction

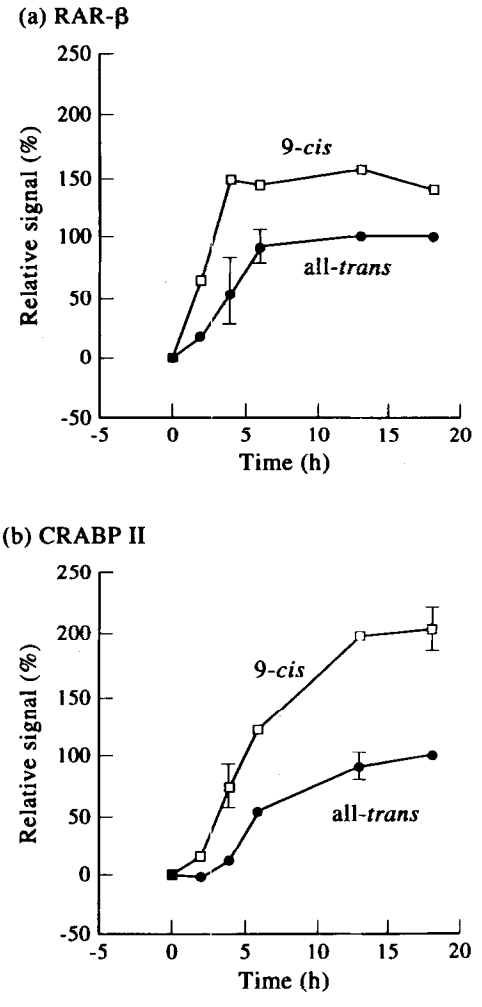

Figure 6. Time course of induction of *RAR-β* and *CRABP II* in SH-SY-5Y cells after treatment with 10^{-7} M all-*trans* or 9-*cis* retinoic acid [44]. (a) Induction of RAR-β (expressed as a percentage of RAR-β signal obtained with 10^{-7} M all-*trans* retinoic acid after 18 h incubation); (b) induction of *CRABP II* (expressed as a percentage of *CRABP II* signal obtained with 10^{-7} M 9-*cis* retinoic acid after 18 h incubation, as above). Error bars: ± S.E.M., $n = 3$. Reprinted with permission of The Biochemical Society and Portland Press from *Biochem J* 1994, 304, 147–154.

reach a plateau 4–6 h after initial exposure to all-*trans* retinoic acid, CRABP II mRNA levels continue to increase for at least 18 h. This difference in induction time-course between RAR-β and CRABP II is attributable, at least in part, to differences in mRNA stability: the RAR-β mRNA half-life (3.9 h) is one-fifth of that for CRABP II [44]. Note that by comparison with human fibroblasts [50], the induction of CRABP II in SH-SY-5Y cells has different characteristics, in that it is insensitive to cycloheximide and CRABP II mRNA levels continue to increase for several hours.

Although 9-*cis* retinoic acid gives induction time courses for *RAR-β* and *CRABP II* that are very similar to those in response to all-*trans* retinoic acid, 9-*cis* consistently produces a greater magnitude of induction for both genes. This is confirmed by dose–response experiments which, in addition, show that these two retinoic acid isomers produce markedly different dose–response curves [44] (Figure 7). As with morphological differentiation, 9-*cis* at low concentrations is less effective than all-*trans*, the response then increasing steeply so that at 10^{-7} M

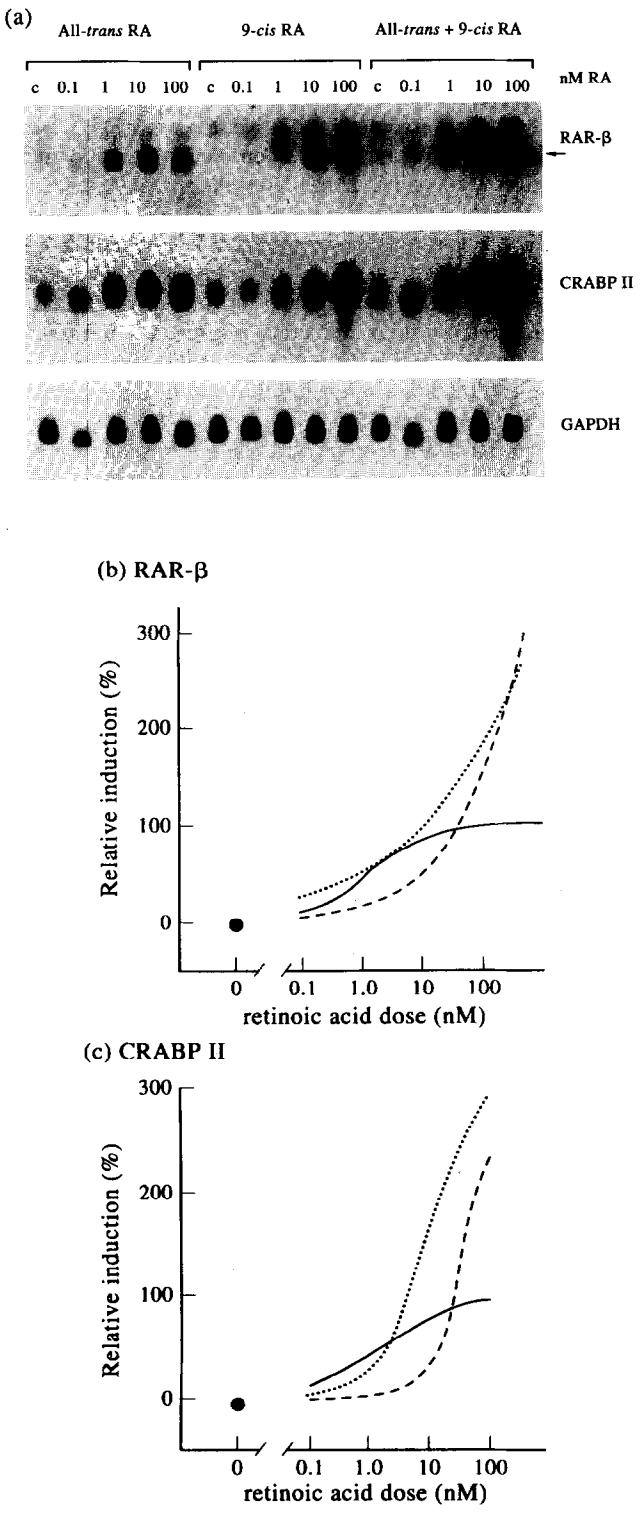

Figure 7. Dose-dependent induction of *RAR-β* and *CRABP II*. (a) Northern blot of RNA extracted from SH-SY-5Y cells after treatment with all-*trans* and/or 9-*cis* retinoic acid at the concentrations indicated for 6 h. c, control ethanol. (b) and (c) Logistic curves fitted to GAPD-corrected data from three experiments [44] for (b) the induction of *RAR-β* (lower transcript, expressed as a percentage of signal obtained with 10^{-7} M all-*trans* retinoic acid, such that control = 0, and 10^{-7} M all-*trans* retinoic acid signal = 100%); and (c) the induction of *CRABP II* (expressed as a percentage of response with 10^{-7} M all-*trans* retinoic acid). ——: all-*trans* retinoic acid; – – –: 9-*cis* retinoic acid; ·····: all-*trans* and 9-*cis* retinoic acid together. Figure 7a is reprinted with permission of the Biochemical Society and Portland Press from *Biochem J* 1994, 304, 147–154.

concentrations, 9-*cis* retinoic acid gives a 3-fold greater induction than all-*trans*. In contrast, all-*trans* retinoic acid is effective at 10^{-10} to 10^{-9} M concentrations with the response reaching a plateau at about 10^{-8} M. The effects of 9-*cis* and all-*trans* retinoic acid together are approximately additive (Figure 7) [44].

The markedly different dose–response curves for the induction of both *RAR-β* and *CRABP II* by all-*trans* or 9-*cis* retinoic acid suggest that a different molecular mechanism is involved. This conclusion is supported by the fact that the induction by all-*trans* is maintained even after the all-*trans* retinoic acid is removed by washout, whereas removal of 9-*cis* retinoic acid from the culture medium after induction results in a decrease in mRNA levels for both RAR-β and CRABP II, consistent with the removal of the transcriptional stimulus at the time of retinoic acid washout [44] (Figure 8).

In clinical trials with retinoic acid, the 13-*cis* isomer has seen greatest use. Previous work has shown that 13-*cis* retinoic acid is 100-fold less potent than all-*trans* in inducing RAR-β expression, at least in murine melanoma cells [49], and this tallies with its lower binding affinity to RARs [52]. In SH-SY-5Y neuroblastoma cells, 13-*cis* retinoic acid is also less potent than either all-*trans* or 9-*cis* and appreciable induction of *RAR-β* and *CRABP II* is apparent only at high concentrations (Figure 9). If the clinical efficacy of 13-*cis* retinoic acid is RAR-mediated, then this is likely to result from isomerisation to all-*trans*. Alternatively, clinical responses could be due to 13-*cis*-specific effects, such as the suicide inhibition of thioredoxin reductase [53], or to non-specific effects such as the disruption of intracellular membranes for which all-*trans* and 13-*cis* retinoic acid are reported to have equal efficiency [54].

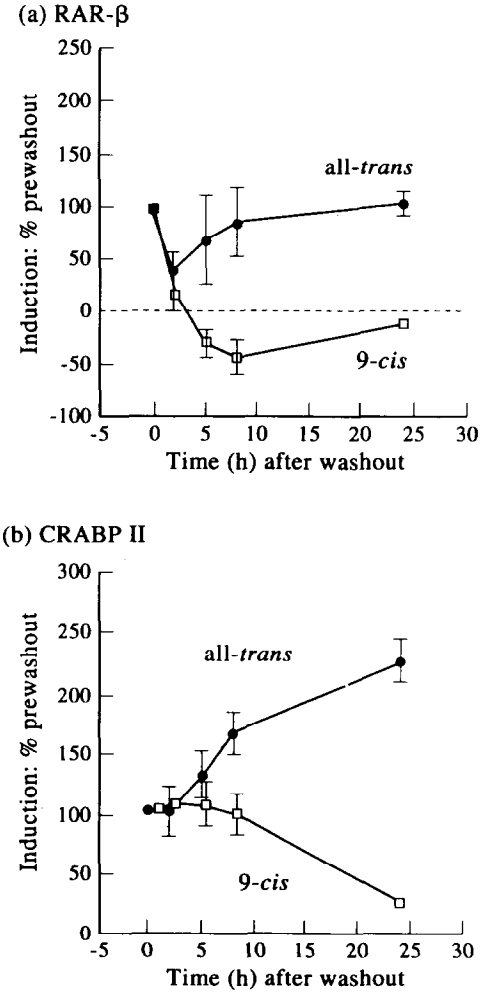

Figure 8. The expression of *RAR-β* and *CRABP II* after induction with all-*trans* or 9-*cis* retinoic acid and subsequent washout, taken from ref. [44]. (a) *RAR-β*: data expressed as a percentage of *RAR-β* signal after treatment with all-*trans* (●) or 9-*cis* (□) retinoic acid for 6 h (washout time zero). i.e. at time zero, the signal for all-*trans* retinoic acid treated cells is 100% (control is 0%, dotted line). Similarly, for 9-*cis* retinoic acid treated cells, the time-zero signal (6 h) is taken as 100%. Note that the level of initial induction by 9-*cis* is higher than for all-*trans* retinoic acid. (b) *CRABP II*, details as for (a). Error bars: ± S.E.M., $n = 3$ or $n = 4$. Reprinted with permission of the Biochemical Society and Portland Press from *Biochem J* 1994, 304, 147–154.

THE MECHANISM OF ACTION OF ALL-*TRANS* AND 9-*CIS* RETINOIC ACID IN THE INDUCTION OF *RAR-β* AND *CRABP* II IN NEUROBLASTOMA CELLS

The interpretation of dose–response curves for 9-*cis* and all-*trans* retinoic acid, and the differential responses to these retinoic acid isomers after induction and washout, is complicated by two factors: the mechanism by which retinoic acid isomers reach the nucleus, and the potential isomerisation of all-*trans* to 9-*cis* and *vice versa*. All-*trans* retinoic acid binds with high affinity (K_d *c.* 4 nM) to cytosolic retinoic acid binding proteins, CRABP I and CRABP II, thought to facilitate its metabolic degradation [55]. 9-*cis* retinoic acid does not show substantial binding to CRABP I or II [56, 57] and, therefore, could be metabolically more stable than all-*trans*. Thus, at low concentrations of 9-*cis* retinoic acid, poor availability to the nucleus may not be an adequate explanation for its lack of activity by comparison with all-*trans*.

The potential for isomerisation is a more serious problem. 9-*cis* retinoic acid is a minor component at isomerisation equilibrium [29, 30], and in SH-SY-5Y cells there can be significant conversion of 9-*cis* to all-*trans*, even after relatively short (6 h) incubation times [44]. At high concentrations of 9-*cis* retinoic acid, some of the responses observed could, therefore, be due to all-*trans* formed by isomerisation. Conversely, all-*trans* retinoic acid is more stable and the induction of *RAR-β* and *CRABP II* by all-*trans* retinoic acid may be due to all-*trans* alone, presumably activating RXR–RAR heterodimers in which the RXR partner acts as a ligand-independent auxiliary factor. Activation at nanomolar concentrations of all-*trans* retinoic acid is consistent with its affinity for RARs, with the final magnitude of response at 10^{-8}–10^{-7} M limited by the intracellular concentrations of RXR–RAR heterodimers. This interpretation is based on the assumption that transcription driven by ligand-activated recep-

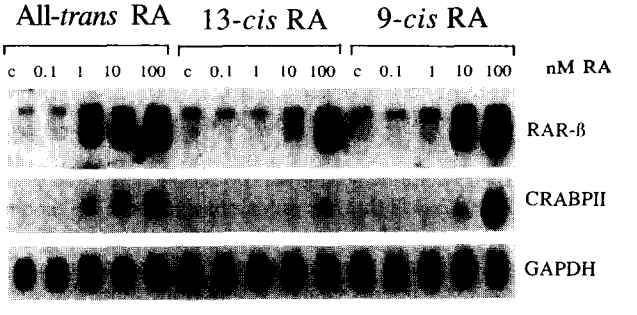

Figure 9. Northern blot showing induction of *RAR-β* and *CRABP II* in SH-SY-5Y cells treated for 6 h with all-*trans*, 13-*cis* or 9-*cis* retinoic acid at the concentrations indicated (Redfern and associates, unpublished data).

tor dimers depends on the affinity of the receptor dimer for its response element (RE) and the affinity of the dimer/RE complex for the basal transcription machinery, TFIID, or associated proteins. Thus, assuming that the availability of transcription factors and RNA polymerase is not limiting, increasing concentrations of ligand-activated receptor dimers will increase the rate of transcription, up to a limit imposed by receptor concentration and physical limits to the rate of transcriptional initiation and elongation.

At low concentrations (10^{-9} M), 9-*cis* retinoic acid is less effective than all-*trans* at inducing *RAR-β* and *CRABP II* [44]. Although 9-*cis* retinoic acid binds to RARs with high affinity [28], this is to a different site within the ligand binding domain to all-*trans* [58]. Nevertheless, 9-*cis* retinoic acid apparently induces similar conformational changes of RARs to all-*trans* [59]. It has been reported that 9-*cis* retinoic acid does not activate RARs [26], but in view of contrary evidence [60], whether or not 9-*cis* activates RARs or the RAR partner of RXR–RAR heterodimers is an issue still to be resolved, but this could be a function of interactions with tissue-specific components of the transcription complex rather than the RARs themselves.

Conversely, at high (10^{-7} M) concentrations, 9-*cis* is 3-fold more effective at inducing both *RAR-β* and *CRABP II* than all-*trans* retinoic acid [44]. Since 9-*cis* retinoic acid is reported to induce RXR homodimer formation cooperatively, at least *in vitro* [61], it is possible that RXR homodimers mediate the response of SH-SY-5Y cells to 9-*cis* retinoic acid. The greater response obtained with high concentrations of 9-*cis* compared with all-*trans* retinoic acid would result either from a greater abundance of RXR protein relative to RARs, or from a higher affinity of either RXR homodimers for the RARE, or of the RXR–RXR/RARE complex for the transcription machinery.

The isomerisation of 9-*cis* to all-*trans* suggests that the greater response to high concentrations of 9-*cis* retinoic acid could be due to synergism between the 9-*cis* and all-*trans* isomers, both binding to RXR–RAR heterodimers. However, since the response to both isomers is approximately additive, and explainable at low concentrations by the all-*trans* isomer alone, such a mechanism may be unlikely. Furthermore, the fact that induction by 9-*cis* retinoic acid is rapidly attenuated by subsequent washout suggests that all-*trans* is not involved in the response, and argues for a distinct mechanism of action of 9-*cis*, perhaps mediated by ligand-dependent RXR homodimers. If this interpretation is correct, data from washout experiments imply that RXR homodimers may be strongly ligand-dependent with a high rate of ligand dissociation. An important caveat is that the maintenance of transcriptional induction after washout of all-*trans* is assumed to result from a low rate of dissociation of the ligand from its receptor and not due to the intracellular release of all-*trans* retinoic acid sequestered by CRABP, a protein which does not bind 9-*cis* [56, 57].

An alternative explanation for the rapid reduction in RAR-β and CRABP II expression after 9-*cis* washout is the possibility that cellular responses to 9-*cis* and all-*trans* retinoic acid are qualitatively different, and that the expression of RAR-β and CRABP II induced by all-*trans* is maintained in its absence by the all-*trans*-dependent synthesis of retinoic acid-independent transcription factors. Furthermore, while we favour the hypothesis that responses to high concentrations of 9-*cis* retinoic acid are mediated by RXR homodimers, we cannot rule out the possibility that induction by 9-*cis* is mediated by its binding to the RAR partner of RXR–RAR heterodimers, particularly since Allenby and associates [62] have found that 9-*cis* has a 6-fold higher rate of dissociation from RAR-γ than does all-*trans* retinoic acid. The roles of different RAR types in the retinoic acid-mediated induction of *RAR-β* and *CRABP II* have not yet been elucidated.

The idea that in SH-SY-5Y cells the induction of *RAR-β* and *CRABP II* by 9-*cis* retinoic acid is mediated by RXR homodimers raises two major issues: (1) how can the possibility of synergism between all-*trans* and 9-*cis* retinoic acid on RXR–RAR heterodimers be evaluated; and (2) do the properties of the RAR-β and CRABP II RAREs support the RXR homodimer interpretation? Synergism between ligands in this context means that the magnitude of transcriptional response to all-*trans* retinoic acid-bound RAR heterodimers is increased by the binding of 9-*cis* to the RXR partner. This implies an increase in affinity for the response element and/or in the affinity of the heterodimer/RARE complex for TFIID or other transcription factors. Evidence for synergism between all-*trans* and 9-*cis* retinoic acid with respect to RXR–RAR-mediated activation of the βRARE is based largely on transient transfection experiments in other cell types [25], but these often suffer from methodological problems, particularly with respect to isomerisation during extended incubations of up to 40 h with all-*trans* or 9-*cis* retinoic acid, and it is difficult to rule out the possibility that, in some cases, *additive* effects of all-*trans* retinoic acid-activated RXR–RAR heterodimers and 9-*cis*-activated RXR homodimers may be mistaken for synergistic activation of RXR–RAR heterodimers by all-*trans* and 9-*cis* retinoic acid.

Evidence for synergy has also been reported for heterodimers of RXRs with vitamin D_3 receptors (VDR) [63], although others have reported that 9-*cis* retinoic acid does not influence the transcriptional activity of RXR–VDR and RXR–thyroid hormone receptor heterodimers [64, 65], and solution dimerisation experiments suggest that 9-*cis* retinoic acid destabilises VDR–RXR heterodimers by promoting RXR homodimer formation [66]. The question of whether 9-*cis* retinoic acid is an activating ligand for the RXR partner of RXR–hormone receptor heterodimers cannot at present be satisfactorily resolved, but it is important to point out that the potential for synergy may not be a function of RXRs but of components of the transcription machinery which interact with the heterodimer or RXR partner. Potential synergy may, therefore, show tissue specificity and dependence on the promoter context of a particular response element.

With respect to RAREs, the βRARE is well characterised and in the presence of 9-*cis* retinoic acid, RXR homodimers will drive transcription from this response element [26, 61]. The same is also true for the DR + 1 type element characteristic of the apoA1 promoter, although the putative DR + 1 RARE associated with the human *CRABP II* gene has not been studied. Thus, the receptor specificities of RAREs *in vitro* support the view that 9-*cis*-activated RXR homodimers mediate gene induction in SH-SY-5Y cells.

GENE EXPRESSION IN RELATION TO NEUROBLASTOMA DIFFERENTIATION AND PROLIFERATION

These studies on SH-SY-5Y cells demonstrate that *RAR-β* and *CRABP II* are rapidly induced by all-*trans* and 9-*cis* retinoic acid, but these isomers apparently act by different mechanisms with 9-*cis* giving a greater response at high concentrations. Furthermore, the relative differentiation-inducing properties of 9-*cis* and all-*trans* retinoic acid are similar to their relative effects on gene induction, but with respect to proliferation, these

isomers have about the same level of activity at high doses. This raises the question of whether or not retinoic acid-induced differentiation and inhibition of proliferation are controlled by different mechanisms. In addition to their role as transcriptional activators, RARs and RXRs can be effective transcriptional inhibitors by binding as ligand-dependent monomers to the c-*fos* and c-*jun* components of the AP1 complex [67], a transcriptional activator involved in controlling cell proliferation [68]. Ligand-dependent but RARE-independent protein–protein interactions between RARs, RXRs and AP1 components could thus account for the similar effects of all-*trans* and 9-*cis* retinoic acid on neuroblastoma cell proliferation.

Although the induction of *RAR-β* and *CRABP II* are early events in retinoic acid-induced SH-SY-5Y cell differentiation, the relevance of these two genes to the differentiation process is unknown. In view of its role in retinoic acid metabolism [55], the induction of *CRABP II* could be a purely homeostatic response by cells programmed to maintain low intracellular levels of retinoic acid. Alternatively, *RAR-β*, as a ligand-dependent transcriptional regulator, could function to regulate subsequent, hypothetical events leading to full morphological and biochemical differentiation. However, although there may well be a link between *RAR-β* expression and differentiation for some cell types, the role of *RAR-β* in neuroblastoma differentiation is unknown. Retinoic acid-induced differentiation has been more extensively studied in EC cells, and these also show induction of *RAR-β*. *RAR-α* is, at least in some cell lines, critically involved in the induction of *RAR-β* and differentiation [69], and RAR-β induction is thought to be a necessary step in *HOX* gene activation during EC cell differentiation [70]. Further work is clearly needed to elucidate the role of *RAR-β* in retinoic acid-induced neuroblastoma differentiation and phenotype.

FUTURE PROSPECTS

9-*cis* retinoic acid may have considerable potential for the treatment of resistant, disseminated neuroblastoma. Recent studies on animal models indicate that 9-*cis* retinoic acid may be more potent that all-*trans* in the suppression of breast cancer [71]. In addition, 9-*cis* retinoic acid is reported to have marked synergistic effects with all-*trans* in promoting the differentiation of retinoic acid-resistant promyelocytic leukaemia cells *in vitro* [72]. Before the potential of 9-*cis* retinoic acid for treating neuroblastoma patients can be fully realised, a greater understanding of the role of different retinoic acid isomers in the control of cell development and differentiation is required. The markedly distinct dose–response properties of 9-*cis* retinoic acid in inducing gene expression and differentiation of neuroblastoma cells raises important issues concerning the mode of action of 9-*cis*, the involvement of RXRs and the effects of 9-*cis* retinoic acid on other hormonally mediated cellular control pathways. A definitive answer to the question of whether RXR homodimers or RAR–RXR heterodimers mediate the effects of 9-*cis* retinoic acid is important for the design of synthetic retinoids active in neuroblastoma, since ligand-dependent RXR-homodimerisation may depend on ligand–receptor interactions not involved in RXR–RAR heterodimerisation [73]. Furthermore, although 9-*cis* retinoic acid is not cytotoxic *in vitro* and no more effective than all-*trans* in inhibiting SH-SY-5Y cell proliferation [16], it will need to be established whether 9-*cis* retinoic acid can be tolerated *in vivo* at high doses. The potential for 9-*cis* to impinge upon vitamin D_3 and thyroid hormone-response pathways may produce unwanted or intolerable side effects when used in young patients. Nevertheless, 9-*cis* retinoic acid and its mechanisms of action suggest new ways to approach the differentiation therapy of neuroblastoma.

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Cytotoxicity of Paclitaxel and Docetaxel in Human Neuroblastoma Cell Lines

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Taxanes are an important new class of anticancer agents that inhibit cell division by the unique mechanism of increasing the rate of microtubule assembly and preventing microtubule depolymerisation. Using the colony inhibition assay, we compared the cytotoxicity of paclitaxel and docetaxel in three human neuroblastoma (NB) cell lines, SH–SY5Y, BE(2)M17 and CHP100. Different exposure times (3, 6, 12, 24, 48 and 72 h) and different concentrations ranging from 0.1 nM to 10 μM were tested. Both paclitaxel and docetaxel show antineoplastic activity in human NB cell lines. Taxanes' antitumour activity varied among the different cell lines, CHP100 being the most sensitive and SH–SY5Y the least sensitive. Paclitaxel cytotoxicity appears schedule-dependent, with marked cell kill observed only for exposures of 24 h or longer. Docetaxel cytotoxicity was dependent upon prolonged exposure only in the SH–SY5Y cell line, while an exposure time of 3–6 h resulted in exponential cell kill in the other two cell lines. Docetaxel was more cytotoxic than paclitaxel with a mean ratio of (paclitaxel/docetaxel) IC_{50} values ranging from 2 to 11. For both taxanes, we observed good correlation between cytotoxic effect and percentage of cells blocked in G2/M phase. A cytotoxic effect occurred at concentrations comparable with those achieved in the plasma of patients treated with these agents in initial clinical trials. The full potential of prolonged infusion or repeated daily administrations of taxanes should be explored in clinical studies, and responses to taxanes in neuroblastoma should be assessed in paediatric phase II studies.

Key words: paclitaxel, docetaxel, neuroblastoma, cell lines, cytotoxicity
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INTRODUCTION

NEUROBLASTOMA (NB) is one of the most common childhood tumours, with the third highest incidence after leukaemia and brain tumours. The prognosis for patients with disseminated disease remains poor. The survival rate for patients of age > 1 year with stage IV disease is generally < 10%, and this has not been modified even with aggressive therapeutic protocols. There is a pressing need to develop new and more effective antineoplastic agents for this disease. Over the past few years, an important new class of microtubule-stabilising agents has been shown to exhibit promising antineoplastic activity. Paclitaxel and docetaxel are the first taxanes that have reached early clinical testing. Paclitaxel was extracted from the bark of the Pacific yew *Taxus brevifolia* about 20 years ago, but its poor availability (only 50–150 mg/kg of dried trunk bark can be isolated) has limited an extensive clinical evaluation. Recently, this problem has been, in part, circumvented by the synthesis of docetaxel, a semisynthetic compound derived from the needles of *Taxus baccata*, by esterification of a non-cytotoxic precursor, the 10-

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