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Retinoid Signalling and Gene Expression in Neuroblastoma Cells: RXR Agonist and Antagonist Effects on CRABP-II and RARβ Expression

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Abstract 9-*cis* Retinoic acid (RA) induces gene expression in neuroblastoma cells more effectively and with different kinetics than other RA isomers, and could be acting in part through Retinoid X Receptors (RXRs). The aim of this study was to characterise the effects of an RXR agonist and RXR homodimer antagonist on the induction of cellular RA binding protein II (CRABP-II) and RA receptor-β (RARβ) in neuroblastoma cells in response to different retinoids. The RXR agonist, LDG1069, was as effective as all-*trans* RA in inducing gene expression, but less effective than 9-*cis* RA. The RXR-homodimer antagonist, LG100754, inhibited the induction of CRABP-II mRNA in SH-SY5Y neuroblastoma cells by 9-*cis* RA or the RXR-specific agonist LGD1069, but had no effect when used with all-*trans* RA. Conversely, LG100754 did not inhibit induction of RARβ mRNA by 9-*cis* or all-*trans* RA, or by LGD1069. RAR- and RXR-specific ligands used together induced CRABP-II and RARβ as effectively as 9-*cis* RA. These results demonstrate the value of combining RXR- and RAR-specific ligands to regulate RA-inducible gene expression. The possibility that RXR-homodimers mediate, in part, the induction of CRABP-II by 9-*cis* RA and RXR-specific ligands is discussed. J. Cell. Biochem. 87: 284–291, 2002.

Key words: 9-*cis* retinoic acid; neuroblastoma; homodimers; RXR; differentiation; proliferation

Retinoic acid (RA) is a ligand for two classes of ligand-dependent transcriptional activators, closely related to steroid and thyroid hormone receptors (TR). These regulate gene transcription by binding as dimers to specific RA response elements (RAREs) associated with gene promoters [Chambon, 1996]. The two classes of retinoid receptors have different ligand binding properties: RA receptors (RARα, RARβ, and RARγ) bind all-*trans* and 9-*cis* RA, whereas retinoid X receptors (RXRα, RXRβ, and RXRγ) bind only 9-*cis* RA. 9-*cis* RA at pharmacological levels has different biological properties and markedly greater effects than all-*trans* RA [Lovat et al., 1994, 1997a; Redfern et al., 1994; Han et al., 1995] and this may stem from that fact that 9-*cis* RA is a ligand for RXRs [Heyman et al., 1992].

In contrast to RARs, RXRs form homodimers and function as heterodimer partners for a variety of nuclear receptors including RARs, vitamin D receptors (VDR), peroxisome proliferator-activated receptors (PPAR), and TR [Stunnenberg, 1993]; in these contexts, RXRs apparently act as ligand-independent auxiliary factors, but act as ligand-dependent transcriptional regulators in the contexts of other receptors such as the NGFI-B (Nur-77)/NURR-1 family [Forman et al., 1995; Perlmann and Jansson, 1995]. In the current paradigm of transcriptional regulation by RXRs, discrimination between specific hormone response elements (HREs) is driven by the RXR dimer

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partner and the number of nucleotides spacing two direct repeats (DR) of the RG(G/T)TCA motif [Stunnenberg, 1993]. For example, RXR-RAR heterodimers bind to and transactivate from a DR5 HRE, whereas RXR homodimers activate transcription from DR1 HREs. Since 9-*cis* RA can bind to RARs [Allegretto et al., 1993], activate RXR homodimers [Zhang et al., 1992] and the RXR partner of NGFI-B/NURR-1 heterodimers [Forman et al., 1995; Perlmann and Jansson, 1995], it can have a range of biological effects not shown by all-*trans* RA which binds to and activates only RARs.

Although 9-*cis* RA is even more effective than all-*trans* (or 13-*cis*) RA as a differentiation agent for neuroblastoma cells in vitro [Lovat et al., 1994; Redfern et al., 1994], the toxicity of 9-*cis* RA in young patients may limit its clinical potential. The existence of different receptors involved in retinoid-response pathways has prompted the search for agonists and antagonists which discriminate between receptor subtypes (RARα, -β, or -γ) and between RARs and RXRs. For example, LGD1069 is an RXR-selective analogue [Boehm et al., 1994], which has considerable anti-tumour activity in animal models of breast cancer [Bischoff et al., 1998] and may have applications in other cancers. To develop less-toxic receptor-specific analogues for clinical use in neuroblastoma, it is important to identify the nuclear-receptor mechanisms of 9-*cis* RA in neuroblastoma cells. The aim of this study was to characterise the effects of an RXR agonist and RXR homodimer antagonist on the induction of cellular RA binding protein II (CRABP-II) and RA receptor-β (RARβ) in neuroblastoma cells in response to different retinoids and to find out if effective induction can be achieved by combinations of RAR- and RXR-specific analogues.

MATERIALS AND METHODS

Retinoids

All-*trans* RA was obtained from Sigma (Poole, UK), 9-*cis* RA was a gift from Michael Klaus (Hoffmann La Roche, Basel), the RAR-specific agonist TTNPB ((*E*)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-I-propenyl]benzoic acid), the RXR-specific agonist LGD1069 (LG100069 Targretin™ 4-[1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydro-2-naphthyl) ethenyl] benzoic acid) and the RXR-homodimer antagonist LG100754 ((2*E*,4*E*,6*Z*)-7-(3-n-propoxy-5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-naphthalen-2-yl)-3-methylocta-2,4,6-trienoic acid) were gifts from Richard Heyman (Ligand Pharmaceuticals, San Diego, CA). All-*trans* or 9-*cis* RA were added to cell cultures from stock solutions in ethanol; other retinoids and analogues were added from stock solutions in dimethyl sulphoxide (DMSO). RA isomers were used at concentrations ranging from 10^{-9} to 10^{-6} M. Receptor-specific analogues/antagonists were used at 10-fold higher concentrations (10^{-8}–10^{-5} M) appropriate to their lower receptor affinity.

Gene Expression Studies

For all experiments, SH-SY5Y cells were grown in culture medium consisting of Dulbecco's modification of Eagle's medium, containing 2 mM L-glutamine and supplemented with 10% foetal calf serum (culture medium), at 37°C in a humidified atmosphere of 5% CO_2 in air. SH-SY5Y cells were seeded into 75 cm^2 flasks (10^5 cells/cm^2 growth area) and allowed to attach overnight. Retinoids, retinoid analogues, or equivalent volumes of ethanol or DMSO as control were added and the cells incubated for 6 h. Total cytoplasmic RNA was extracted, size-fractionated by electrophoresis through 1.2% agarose/formaldehyde gels and transferred by vacuum blotting to nylon membranes [Redfern et al., 1994]. Membranes were probed consecutively with ^{32}P-labelled cDNA probes for CRABP-II, RARβ, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [Redfern et al., 1994], exposed to a phosphorimager screen and scanned on a Molecular Dynamics phosphorimager (Amersham Biosciences, Little Chalfont, UK).

Data Analysis

Data were analysed using the Systat statistical package, version 10 (SPSS, Inc.). For each Northern blot, CRABP-II, and RARβ phosphorimager signal intensities were corrected for loading (GAPDH signal), expressed as a proportion of the relevant GAPDH-corrected signal for control, uninduced cells, and scaled relative to the induction obtained using 10^{-7} or 10^{-6} M (depending on experiment) 9-*cis* RA, which was regarded as the reference and set at 100%. Scaling the response in this way allowed variation in responses between experiments with different batches of cells to be accounted for. Values for the percent relative induction in

different experiments were combined to obtain mean induction for the respective cell treatments. Data were normally distributed (Kolmorogorov-Smirnov test, Lillefors modification, $P > 0.25$), but since the variance increased in proportion to the mean, statistical analyses were done on log-transformed data. The data for the effects of the RXR antagonist, LG100754, were analysed by a separate ANOVA for each retinoid (9-*cis* RA, all-*trans* RA, and LGD1069) with and without antagonist at two concentrations (four treatment groups in total for each of 9-*cis* RA, all-*trans* RA, and LGD1069). Hypothesis tests using pooled variance from the ANOVA were used to compare responses with and without the antagonist. Since responses were scaled relative to response at one concentration of 9-*cis* RA, for analysis of the effects of LG100754 on 9-*cis* RA response, three treatments groups were entered in the ANOVA and the effects of the antagonist at the reference 9-*cis* RA concentration were tested against an expected mean of 100%. ANOVA and hypothesis tests were also used to compare the effects of different retinoids (all-*trans* RA, LGD1069, TTNPB, and LGD1069 with all-*trans* RA or TTNPB) against the expected mean of 100% for 9-*cis* RA. Error bars are ± SEM as specified in the figure legends, or the range when sample size (n) = 2.

RESULTS

Induction of CRABP-II Expression by RXR Agonists and Antagonists

The RXR-specific ligand LGD1069 induced the expression of CRABP-II mRNA with similar dose–response characteristics to all-*trans* RA, but induction did not reach the levels achieved with 10^{-7} M 9-*cis* RA (Fig. 1). Previous studies have shown that 9-*cis* RA has distinct dose–response characteristics with respect to the induction of CRABP-II and RARβ and it has been suggested that the effects of high concentrations of 9-*cis* RA in neuroblastoma cells might be mediated by RXR-homodimers [Redfern et al., 1994]. To test this, SH-SY5Y cells were treated for 6 h with 9-*cis* RA (10^{-8} and 10^{-7} M), all-*trans* RA (10^{-8} and 10^{-7} M) and LGD1069 (10^{-7} and 10^{-6} M), in the presence or absence of the RXR-homodimer antagonist LG100754 (10^{-7} or 10^{-6} M, respectively). Although reported to have partial RXR agonist activity in the context of RXR–RAR heterodimers [Lala et al., 1996], LG100754 on its own did not induce expression of CRABP-II or RARβ in SH-SY5Y cells (Figs. 2 and 4). However, LG100754 markedly inhibited the induction of CRABP-II by 10^{-7} M 9-*cis* RA (ANOVA $F_{1,11} = 15.8$, $P = 0.002$) or 10^{-6} M LGD1069 (ANOVA $F_{3,15} = 3.84$, $P = 0.03$; hypothesis test 10^{-6} M LGD1069 vs. 10^{-6} M LGD1069 plus 10^{-6} M LG100754, $F_{1,15} = 7.9$, $P = 0.013$) but had no significant effect on the lower induction of CRABP-II in response to 10^{-8} M 9-*cis* RA or 10^{-7} M LGD1069 ($F_{1,11} = 0.036$, $P > 0.8$, and $F_{1,15} = 2.6$, $P = 0.13$, respectively) (Figs. 2 and 3). LG100754 also had no inhibitory effect on the induction of CRABP-II in response to either concentration of all-*trans* RA (ANOVA $F_{3,16} = 1.44$, $P = 0.3$) (Figs. 2 and 3).

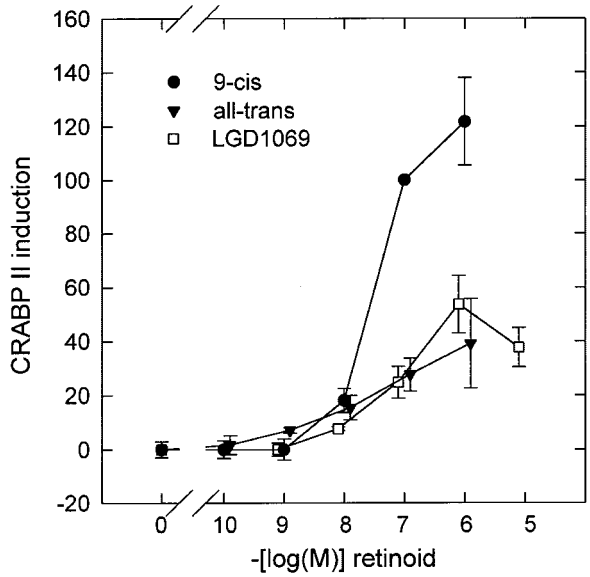


Fig. 1. Dose–response curves for the induction of CRABP-II mRNA in SH-SY5Y neuroblastoma cells after treatment for 6 h with 9-*cis* RA (●), all-*trans* RA (▼) or the RXR-selective analogue LGD1069 (□). Points are mean induction ± SEM (n = 3–6, except 10^{-6} M 9-*cis* RA where n = 2), corrected for RNA loading using the GAPDH signal and scaled relative to control, vehicle-treated (> 0.1% ethanol or > 0.1% DMSO) cells (0% induction) and cells treated with 10^{-7} M 9-*cis* RA (100% induction).

Induction of RARβ Expression by RXR Agonists and Antagonists

LGD1069 was less effective at inducing RARβ than either 9-*cis* or all-*trans* RA (Fig. 4), and these results are comparable to data reported earlier using LGD1069 and a different RXR-specific ligand [Lovat et al., 1997b; Irving et al., 1998]. In contrast to its effects on CRABP-II induction, LG100754 had no significant effect on the induction of RARβ by 9-*cis* RA (Overall

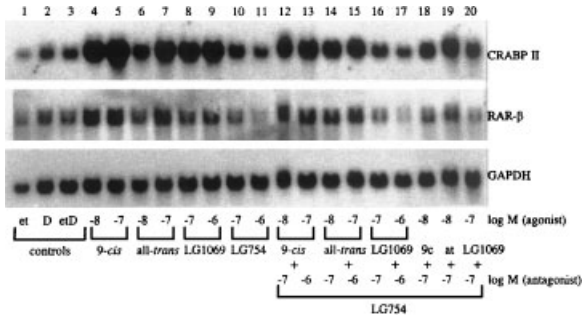


Fig. 2. Northern blot of RNA from SH-SY5Y cells treated with retinoid agonists and antagonists. The blot was probed successively with cDNA probes for CRABP-II, RARβ, and GAPDH (RNA loading control). et, ethanol control; D, DMSO; etD, ethanol and DMSO; 9-*cis* or 9c, 9-*cis* RA; all-*trans* or at, all-*trans* RA; LG1069, RXR-specific agonist LGD1069; LG754, RXR-homodimer antagonist LG100754. Ligand concentrations are given in the figure.

ANOVA $F_{2,8} = 2.8$, $P > 0.12$, hypothesis tests $F_{1,8} < 1.5$, $P > 0.25$). Similarly, LG100754 also had no significant effect on the induction of RARβ in response to all-*trans* RA (ANOVA $F_{3,10} = 1.07$, $P > 0.4$) or LGD1069 (ANOVA $F_{3,9} = 0.3$, $P > 0.8$) (Figs. 2 and 4).

Induction of CRABP-II and RARβ by RAR- and RXR-Specific Ligands in Combination

To compare the properties of RAR- and RXR-specific ligands with respect to the induction of CRABP-II and RARβ, SH-SY5Y cells were treated for 6 h with either 9-*cis* RA, LGD1069, or the RAR-specific ligands TTNPB [Pignatello et al., 1997] or all-*trans* RA. Under these conditions, all-*trans* RA was less effective at inducing CRABP-II (Fig. 5), consistent with previous data [Redfern et al., 1994]. LGD1069 and TTNPB also induced CRABP-II but to a lower level than 9-*cis* RA (ANOVA $F_{4,20} = 9.76$, $P < 0.001$; LGD1069 and TTNPB tested against a mean of 100%, $F_{1,20} = 5$ and 34.15, $P = 0.037$ and < 0.001, respectively). Treatment of cells with the RXR-specific ligand LGD1069 together

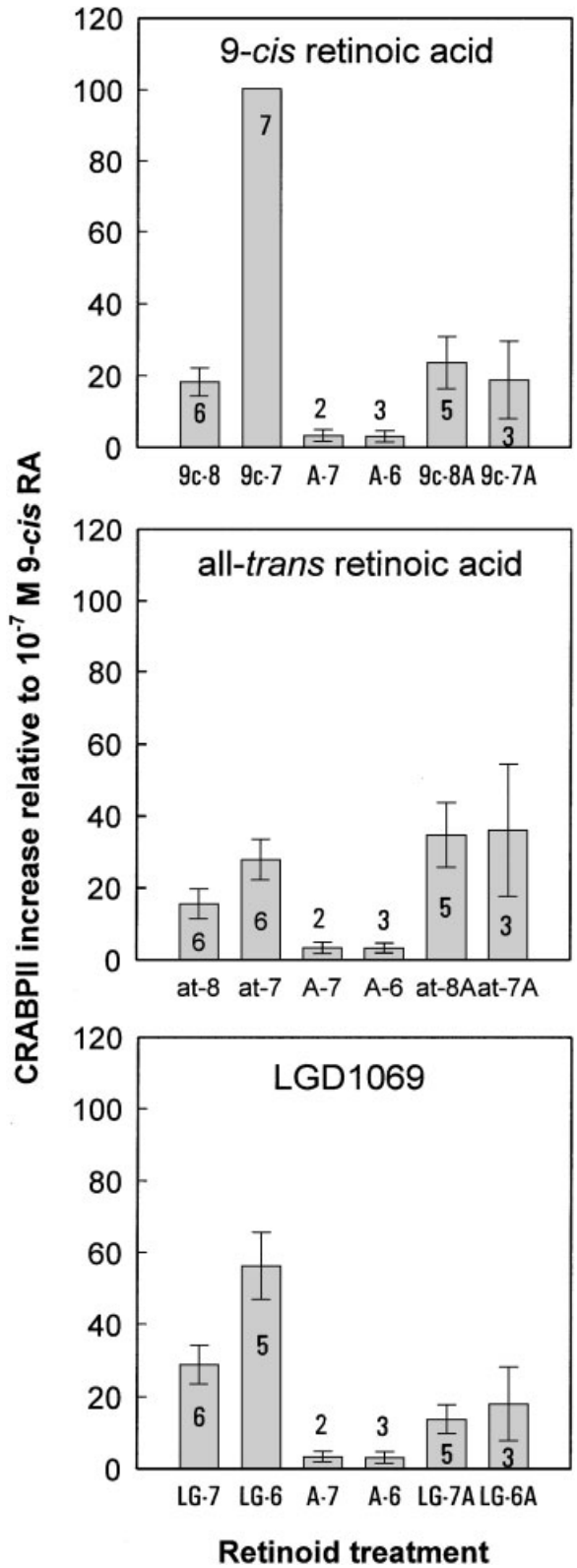
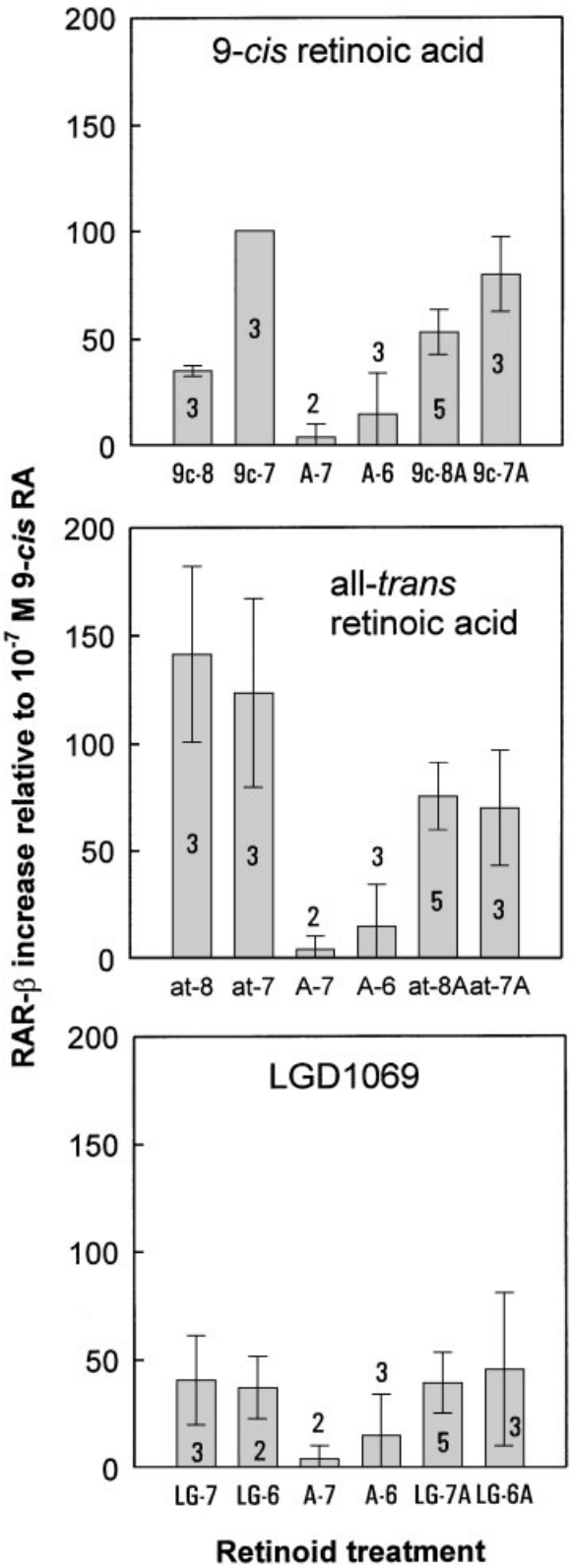


Fig. 3. Effect of RXR-homodimer antagonist LG100754 (10^{-7} and 10^{-6} M) on CRAPB II induction in response to 9-*cis* RA (**A**, 10^{-8} and 10^{-7} M, respectively), all-*trans* RA (**B**, 10^{-8} and 10^{-7} M, respectively), and LGD1069 (**C**, 10^{-7} and 10^{-6} M, respectively). Bar heights are mean induction ± SEM, corrected for RNA loading using the GAPDH signal and scaled relative to control, vehicle-treated (> 0.1% ethanol or > 0.1% DMSO) cells (0% induction, n = 7) and the response of cells treated with 10^{-7} M 9-*cis* RA (100% induction). The number within (or near) each bar is the sample size. Abbreviations: 9c, 9-*cis* RA; A, RXR-homodimer antagonist LG100754; at, all-*trans* RA; LG, RXR-specific agonist LGD1069.



with the RAR-specific ligands all-*trans* RA or TTNPB resulted in a marked induction of CRABP-II mRNA. Although LGD1069 with all-*trans* RA appeared to give a better response than LGD1069 with TTNPB, with the present sample size there was no significant difference in mean response between these treatments ($F_{1,20} = 1.057, P = 0.316$), and both were not significantly different from the response obtained with 9-*cis* RA alone ($F_{1,20} = 3.92, P = 0.062$, and $F_{1,20} = 0.007, P > 0.9$, for LGD1069 with all-*trans* RA or TTNPB, respectively, tested against a mean of 100%). However, the result for LGD1069 with all-*trans* RA approached statistical significance, suggesting that this retinoid combination may be more effective than 9-*cis* RA (Fig. 5). Overall, similar results were obtained for RARβ although the sample sizes were smaller for these experiments (Fig. 5).

DISCUSSION

In contrast to its minimal effects on SH-SY5Y cell differentiation and proliferation [Campbell Hewson et al., 2000], LGD1069 was an effective inducer of CRABP-II and RARβ expression, although less effective on its own than 9-*cis* RA but as effective in combination with RAR-selective retinoids. The differential effects of the RXR-homodimer antagonist LG100754 on the induction of CRABP-II and RARβ suggests that these two genes are regulated in different ways by 9-*cis* RA and LGD1069. There are two main issues to resolve with respect to the interpretation of these data: the possible mechanisms of 9-*cis* RA and RXR-selective retinoids in the induction of gene expression, and whether RXR homodimers have a physiological role in retinoid responses and particularly the induction of CRABP-II.

The induction of CRABP-II in SH-SY5Y neuroblastoma cells by 9-*cis* RA has distinct dose–response characteristics: a poor response at low concentrations (10^{-9} M) increasing steeply to a

Fig. 4. Effect of RXR-homodimer antagonist LG100754 (10^{-7} and 10^{-6} M) on RARβ induction in response to 9-*cis* RA (**A**, 10^{-8} and 10^{-7} M, respectively), all-*trans* RA (**B**, 10^{-8} and 10^{-7} M, respectively), and LGD1069 (**C**, 10^{-7} and 10^{-6} M, respectively). Bar heights are mean induction ± SEM, corrected for RNA loading using the GAPDH signal and scaled relative to control, vehicle-treated (> 0.1% ethanol or > 0.1% DMSO) cells (0% induction), and cells treated with 10^{-7} M 9-*cis* RA (100% induction). Abbreviations are as in Figure 3. The number within (or near) each bar is the sample size.

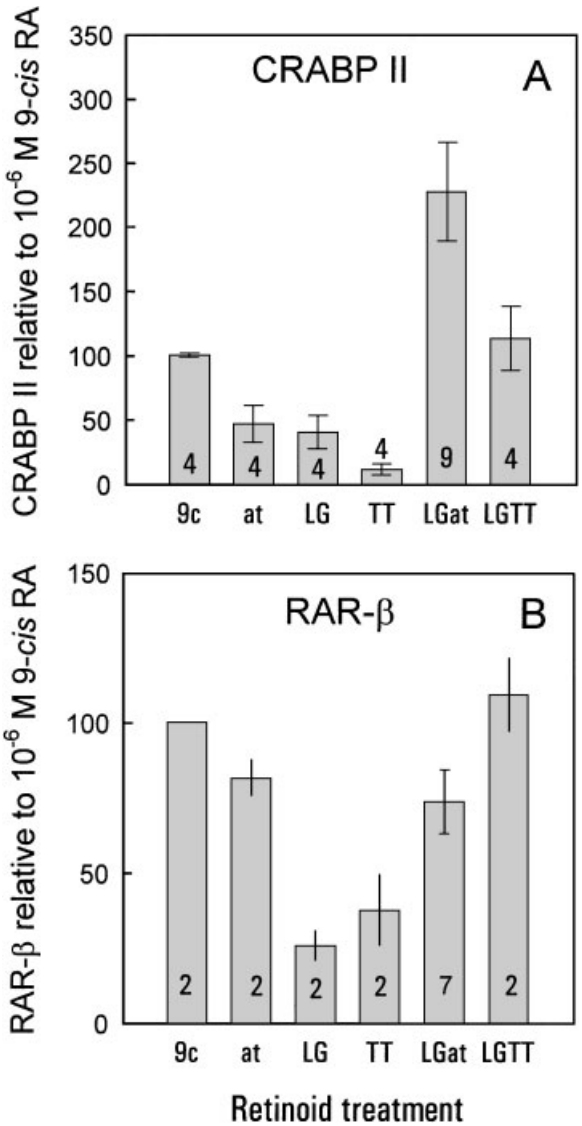


Fig. 5. Induction of CRABP-II (**A**) and RARβ (**B**) by combinations of RAR- (all-*trans* RA or TTNPB) and RXR-selective (LGD1069) agonists. Bar heights are mean induction ± SEM in A and mean ± range in B, corrected for RNA loading using the GAPDH signal and scaled relative to control, vehicle-treated (> 0.1% ethanol or > 0.1% DMSO) cells (0% induction) and cells treated with 10^{-6} M 9-*cis* RA (100% induction). The number within (or near) each bar is the sample size. Abbreviations: 9c, 9-*cis* RA; at, all-*trans* RA; LG, LGD1069; TT, TTNPB; LGat, LGD1069 + all-*trans* RA; LGTT, LGD1069 + TTNPB.

large response at concentrations of 10^{-7} M or greater [Redfern et al., 1994]. These characteristics echo the co-operativity that has been previously described for RXR-homodimer formation or activity [Zhang et al., 1992], most recently characterised as a co-operative dissociation of RXR tetramers [Kersten et al., 1995b; Chen et al., 1998a]. Extensive biochemical data now show that RXRα forms tetramers in solution which dissociate in the presence of ligand (9-*cis* RA or LGD1069) to RXR homodimers and monomers [Kersten et al., 1995a, 1997a,b; Chen et al., 1998b]. In the presence of RARs, the relative binding affinities of RXR to an RAR or RXR partner greatly favours the formation of RXR–RAR heterodimers [Dong and Noy, 1998]. The presence of RXR and/or RAR ligands alter RXR–RAR binding affinities such that, relative to the presence of RAR ligands, RXR ligands alone (such as LGD1069) reduce interactions between RXRs and RARs, whereas the presence of both RAR and RXR ligands increases binding of RXRs to RARs twofold [Dong and Noy, 1998]. Thus, according to this model, 9-*cis* RA will co-operatively induce RXR tetramer dissociation, and since 9-*cis* RA will isomerise to all-*trans* RA within the cells, even after 6-h incubations [Redfern et al., 1994], the presence of RARs and ligands to both RXRs (9-*cis* RA) and RARs (all-*trans* RA) will favour the generation of maximal levels of RXR–RAR heterodimers. Clearly, under this scenario, increased availability of RXRs resulting from treatment of cells with 9-*cis* RA (but not all-*trans* RA which is not an RXR ligand) will lead to increased transcription from DR5 RAREs, and could be an adequate explanation for the dose–response kinetics of induction of CRABP-II and RARβ in response to 9-*cis* RA [Redfern et al., 1994]. Since LGD1069 will also induce RXR tetramer dissociation [Kersten et al., 1997a], a similar mechanism will explain the observation that the RXR-selective ligand in combination with RAR-selective ligands (all-*trans* RA or TTNPB) is as least as effective as 9-*cis* RA in inducing CRABP-II and RARβ.

Clearly, the high level of induction of CRABP-II and RARβ by 9-*cis* RA, or LGD1069 in combination with RAR-specific retinoids, does not require the participation of RXR homodimers, but there are two outstanding issues: how does an RXR selective ligand, LGD1069, induce CRABP-II and RARβ expression on its own, and why does LG100754 apparently inhibit 9-*cis* RA- or LGD1069-induced CRABP-II expression but not RARβ expression? With respect to the activity of RXR-selective retinoids on their own, since such RXR-selective retinoids induce RXR tetramer dissociation [Kersten et al., 1997a], it is possible that LGD1069-activated heterodimers between RXRs and other nuclear receptors such as NURR-1 or NGFI-B (Nur-77) [Perlmann and Jansson, 1995] drive CRABP-II

and RARβ expression through DR5 RAREs in these cells. In S91 melanoma cells, there is evidence that RXR-selective ligands promote occupancy of the RARβ DR5 RARE [Ikeda et al., 1998].

The question whether RXR homodimers play a role in gene expression in SH-SY5Y neuroblastoma cells is more difficult to assess. Despite the results with LG100754 that argue for a role of RXR-homodimers in CRABP-II induction, we remain doubtful over the role and physiological existence of RXR-homodimers as ligand-dependent regulators of gene expression in SH-SY5Y neuroblastoma cells; we have not been able to find evidence from electrophoretic mobility shift experiments to support the idea that either RXRs or RXR homodimers regulate CRABP-II gene expression in these cells [Rana et al., 2002]. Although described as an RXR homodimer antagonist, LG100754 also has other properties: it can display RAR agonist effects [Lala et al., 1996] (but not with respect to CRABP-II and RARβ expression in SH-SY5Y cells), RXR agonist effects in the context of RXR–PPAR heterodimers [Cesario et al., 2001], and also 'sensitise' RXR–PPARγ heterodimers to PPARγ ligands [Forman, 2002]. Neuroblastoma cells can respond to PPARγ agonists [Han et al., 2001], and it is conceivable that ligand-activated RXR–PPARγ heterodimers could be repressors (rather than activators) of CRABP-II via the DR1 RXRE [Astrom et al., 1992] and that this function could be activated by endogenous ligands after sensitising with LG100754. The fact that CRABP-II, but not RARβ, has a DR1 RXRE may be the key to the specific effects of LG100754 on CRABP-II expression. However, in the absence of convincing mobility-shift data to support a role of RXR homodimers in these cells [Rana et al., 2002], and since LG100754 is relatively uncharacterised with respect to its effects on RXR–RAR interactions in heterodimers and RXR tetramers, it would be premature to conclude that the ability of LG100754 to inhibit RXR-ligand-induced expression of CRABP-II is a function of RXR homodimer antagonism.

This study shows that RXR-selective retinoids in combination with RAR-selective compounds can be effective modulators of gene expression in neuroblastoma cells. However, this is in marked contrast to the relatively poor effects, compared to 9-*cis* RA, of such ligand combinations on the differentiation and proliferation of these cells [Campbell Hewson et al., 2000]. Clearly, the identification of the main differentiation-regulating genes of neuroblastoma cells is critical for an assessment of how retinoids can be used to mimic the favourable in vitro properties of 9-*cis* RA. The response of different genes to retinoids will depend on a number of factors, particularly the receptor types and abundance available for receptor dimerization and ligand activation, the gene-specific repertoire of response elements, and the expression of gene- and cell-type-specific co-regulators and competing transcription factors. Detailed studies of the quantitative binding and activation relationships underlying different receptor–receptor and receptor–ligand interactions are required to model the way in which different receptor-selective ligands can be used to modify gene expression and cell behaviour.

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