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Selective High Affinity Retinoic Acid Receptor α or β-γ Ligands

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SUMMARY

Biological effects of retinoic acid (RA) are mediated through its binding to three closely related nuclear receptors (RARα, RARβ, and RARγ) belonging to the steroid-thyroid nuclear receptor family. RARs are able to modulate the transcription of specific genes by binding to responsive elements located in the promoter-enhancer region of these genes. As demonstrated by *in situ* hybridization, the distribution of each RAR type in the developing embryo, as well as in the adult, is not uniform. In this context, synthetic retinoids that would behave as selective ligands would be invaluable for studying the respective roles of each RAR type in cultured cells, whole animals, and embryos. Moreover, from a pharmacological point of view, such selective compounds may possess a higher therapeutic index and a lower teratogenic risk, because they might affect specific tissues and spare some others. As an approach to this problem, we have set up two complementary assays, (i) an *in vitro* binding assay to determine the K_d values of retinoids for RARα, RARβ, and RARγ and (ii) a functional assay in cultured cells to evaluate the potential of retinoids to transactivate, through their binding to one type of RAR, a reporter gene. The binding assay uses nuclear extracts of COS-7 cells transfected with vectors expressing RARα, RARβ, or RARγ. The functional assay is a measure of chloramphenicol acetyltransferase (CAT) activity in HeLa cells co-transfected with the expression vectors used in the binding assay and the reporter gene TRE-tk-CAT. Selective agonists for RARα (Am80 and Am580) and RARβ-RARγ (CD495 and CD564) were identified. However, compounds with pure RARβ or RARγ selectivity have not yet been identified.

RA is a morphogenetic compound involved in vertebrate development (1–6). it plays a major role in the differentiation and maintenance of several tissues, including skin (7–10), cartilage (11), and the hematopoietic and nervous systems (12, 13). The effects of RA and synthetic retinoids are mediated by nuclear receptors termed RARs (14, 15), which belong to the steroid-thyroid hormone receptor family. Three subtypes, namely RARα, RARβ, and RARγ, have been identified so far (16–22). In addition, another subfamily of nuclear receptors (termed RXRs), which might mediate some of the effects of RA or its different metabolites, has been described recently (23).

The binding of RARs to specific responsive elements (RA-responsive elements) and to other related hormone-responsive elements (TREs and VDREs) located in the promoter regions of the target genes leads to a positive or negative modulation of the transcription of these genes (21, 24–29). Subsequently, the integration of all these genetic responses (pleiotropic control) results in the expression of a distinct phenotype of the target cells. It is still not known whether the various RARs exhibit distinct selectivity towards the responsive elements of each particular gene or whether they differ from one another in the amplitude of the transcription modulation they can elicit, but their distribution in the different tissues of the embryo and the adult is not uniform. For instance, hematopoietic cells express exclusively RARα messages (30), whereas RARβ transcripts are particularly abundant in brain and areas undergoing programmed cell death (31), and RARγ transcripts in skin and cartilage (21, 22, 32).

In this context, it is obvious that the identification of synthetic retinoids selective for each RAR subtype would be invaluable for studying, in whole embryos or animals or in cultured cells, the respective contribution of each RAR subtype in the biological effects of RA. Moreover, from a pharmacological point of view, selective compounds may possess a higher therapeutic index, because they might target a restricted number of tissues (for instance, skin for RARγ, leukemia cells for RARα) but spare other tissues, including some of the embryo. Thus, it can be expected that, among selective retinoids, some would exhibit a lower teratogenic risk and produce fewer side effects.

In the present work, we have combined two assays on retinoids, (i) an *in vitro* binding assay for the determination of the K_d values of retinoids for RARα, RARβ, and RARγ and (ii) a

ABBREVIATIONS: RA, retinoic acid; RAR, retinoic acid receptor; DMSO, dimethyl sulfoxide; AC_{50}, 50% activating concentration; PBS, phosphate-buffered saline; CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco's modified Eagle's medium; TRE, thyroid hormone-responsive element; VDRE, vitamin D responsive element; HPSEC, high performance size exclusion chromatography; CRABP, cytosolic retinoic acid-binding protein.

functional assay in cultured cells for the evaluation of the capacity of retinoids to transactivate a reporter gene after their interaction with RARα, RARβ, or RARγ. So far, we have identified compounds with RARα selectivity (Am80 and Am580), compounds with RARβ-RARγ selectivity (CD495 and CD564), and compounds with no selectivity (CD367).

Materials and Methods

Chemicals

The chemical syntheses of the retinoids tested have been described elsewhere (Am80 and Am580, Ref. 33; CD367, CD495, and CD564, Refs. 34–36). Their formulas and nomenclature are indicated in Fig. 1. CD367, used as the labeled ligand in competition experiments, has been tritiated in our institute with a specific activity of 52.8 Ci/mmol (34, 35). RA was purchased from Interchim (Montluçon France).

Binding Assay

All binding assays were performed on nuclear extracts of COS-7 cells transfected with the vectors expressing RARα (16), RARβ (19), or RARγ (purchased from La Jolla Cancer Research Foundation), as described (37).

Production of RARα, RARβ, and RARγ. COS-7 cells, grown in DMEM supplemented with 10% fetal calf serum, were transfected with the vectors expressing RARα, -β, and -γ, using the Polybrene (38) technique (10^5 cells/10 μg of cDNA/dish). After a transfection period of 26 hr, the cells were partially permeabilized by a 4-min incubation in culture medium containing 20% DMSO, washed three times with PBS, and re-fed with standard medium for an additional 66-hr period. Cells were harvested, suspended in binding buffer (20 mM Tris·HCl, pH 7.2, 1 mM EDTA, 2 mM dithiothreitol, 50 mM NaCl, 0.3 mM phenylmethylsulfonyl fluoride), and freeze-dried for 24 hr.

Receptor preparation. All experiments were carried out at 4°. Cell lyophilisates were resuspended in distilled water, which led to complete cell lysis. Cytosol was extracted by a 5-min centrifugation at 10,000 × *g*. The resulting pellet contained the nuclei, as shown by electron microscopic examination. The nuclei were washed once with binding buffer and then subjected to a DNase I digestion, followed by high salt extraction (0.4 M NaCl). A nuclear extract was obtained by a second centrifugation step (5 min, 10,000 × *g*). Protein contents of the cytosol and nuclear extracts were determined using the Bradford assay.

Binding assay and HPSEC separation. Saturation experiments were performed by mixing aliquots of cytosol or nuclear extracts (98 μl) with 2 μl of increasing concentrations of [^{3}H]CD367 in DMSO. For competition experiments, aliquots of nuclear extracts (96 μl) were incubated with a fixed concentration of [^{3}H]CD367 at 1.9 nM (2 μl) and increasing concentrations of unlabeled competitor in DMSO (2 μl). After a 2-hr incubation at 4°, 50 μl of the incubation mixture were subjected to HPSEC separation on a GF250 column (250 × 9.4 mm; DuPont de Nemours) and eluted with 0.3 M KH_2PO_4, pH 7.8, at a flow rate of 1 ml/min. The column was standardized with a mixture containing egg albumin (45 kDa) and myoglobin (17.7 kDa). After a lag period of 5 min, 28 fractions of 0.3 ml were collected and counted in a liquid scintillation counter, using 10 ml of Picofluor 30 (Packard) as scintillation liquid. Radioactive counts obtained in fractions containing the RAR-CD367 complex (fractions 10–15) or the CRABP-CD367 complex (fractions 16–20) were added and expressed as picomoles of bound ligand per milligram of protein. The total concentration of the ligand in the assay was quantified by counting a 20-μl sample of the incubation mixture. The saturation and competition curves were analyzed by computer-assisted nonlinear regression analysis. K_d values are expressed as the mean value of two or three independent determinations.

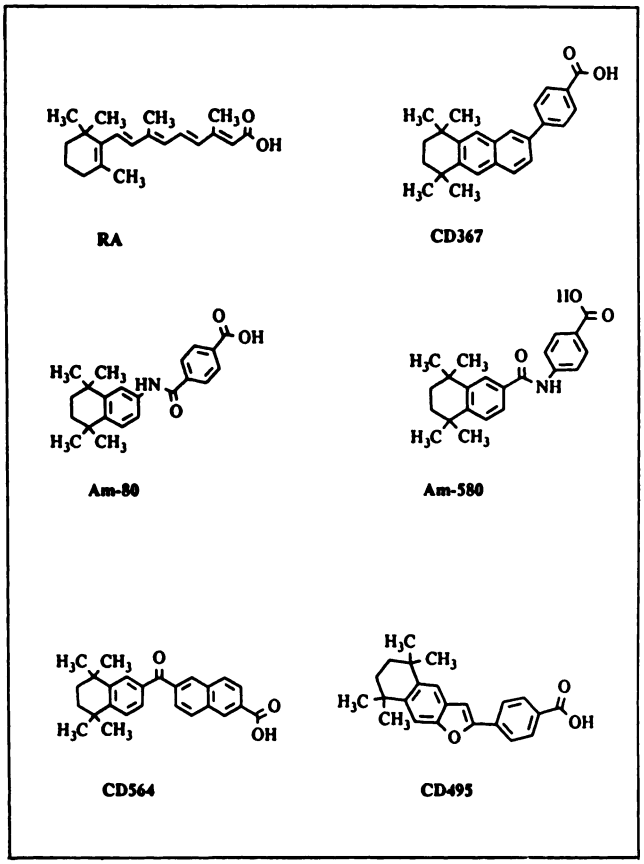

Fig. 1. Formulas of RA and of synthetic retinoids. CD367, 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-anthracenyl)benzoic acid; Am80, 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenylcarbamoyl)benzoic acid; Am580, 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthamido)benzoic acid; CD564, 6-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthoyl)-2-naphthoic acid; CD495, 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphtho[2,3-*b*]furan-2-yl)benzoic acid.

Functional Transactivation Assay

HeLa cells were grown in DMEM containing 10% fetal calf serum. The day before transfection, cells were seeded on 100-mm tissue culture dishes at a density of 5 × 10^5 cells/dish. HeLa cells were co-transfected, using the calcium phosphate co-precipitation technique (39), with 5 μg of TRE_3-tk-CAT reporter plasmid (21), together with 2 μg of expression vectors for α, β, or γ human RARs and 5 μg of pRSVlacZ as an internal control. Eighteen hours after transfection, cells were washed twice in PBS and re-fed with medium (DMEM with 10% delipidized fetal calf serum) containing different concentrations of retinoids dissolved in DMSO or just DMSO, as a control. Twenty-four hours later, the cells were washed twice with PBS and directly lysed in the dish by one freeze-thaw cycle in the presence of 1 ml of lysis buffer (100 mM Tris·HCl, pH 7.8, containing 1 mM $MgCl_2$). CAT activity was determined by the NEN fluor diffusion assay NET-290L (40), normalized to β-galactosidase activity, and expressed as percentage of maximal induction at each retinoid concentration, after background CAT activity had been substracted. Two to four dose-response curves were performed. Each experimental point was performed in triplicate. The concentrations of retinoid giving 50% of maximal activation (AC_{50}) were determined from the plots.

Results

***In vitro* binding assay.** We first characterized the binding properties of the reference compound CD367 for RARα, RARβ,

and RARγ, by performing full saturation experiments with tritiated CD367 (Fig. 2). K_d values, calculated by computer-assisted nonlinear regression analysis, were found to be 3 nM for RARα, 4 nM for RARβ, and 1.5 nM for RARγ. Thus, these binding data show that CD367 behaves as a nonselective high affinity ligand for the three types of receptors. Fig. 2 also shows Scatchard plots corresponding to each receptor type. The K_d values determined by this method were similar to those determined by nonlinear regression analysis. Table 1 reports the K_d values as well as the standard deviations, the correlation coefficients, and the number of binding sites in the extract and per cell. If we assume that nontransfected COS cells contain <500 sites/cell (41), then the transfection results at least in a 40-fold increase in the number of binding sites (see Table 1). The

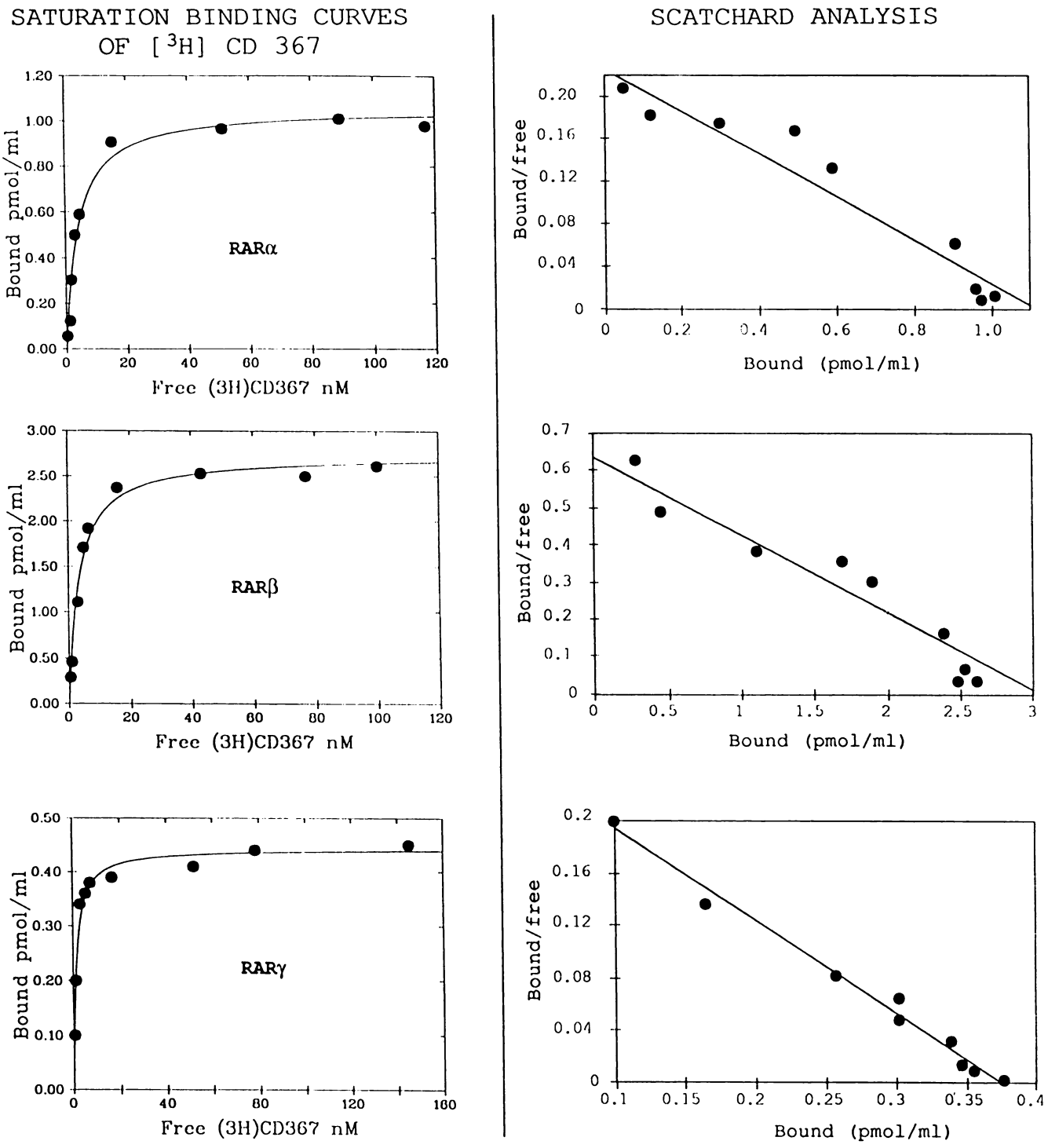

Fig. 2. *Left*, Saturation binding curves of [^{3}H]CD367 on nuclear RARα, RARβ, and RARγ obtained after transfection of COS cells. *Right*, corresponding Scatchard plots.

TABLE 1

K_d values and number of binding sites for [^{3}H]CD367 in RARα, RARβ, and RARγ preparations, determined using the Scatchard representation

n represents the number of independent experiments. The standard deviation and the correlation coefficient correspond to 95% confidence limits. The determination of the Hill number shows a single class of sites.

[^{3}H]CD367	RARα	RARβ	RARγ
Scatchard analysis			
K_d (nM)	3 ± 1 (n = 3)	4 ± 1 (n = 3)	1.5 ± 0.5 (n = 4)
Correlation coefficient	0.96	0.97	0.99
B_{max} (pmol/ml)	1	2.7	0.37
B_{max} (pmol/mg of protein)	1	0.9	0.8
Number of sites/cell	23,000	20,000	19,000
Hill representation			
Hill number	0.98	1.02	0.93
Correlation coefficient	0.98	0.99	0.99

endogenous receptors must then be considered negligible, for the K_d determinations. Moreover, Hill plots (Table 1) are indicative of a single population of sites for RARα, RARβ, or RARγ.

Because of its binding properties and its chemical stability, [^{3}H]CD367 was used as the labeled ligand in competition experiments using unlabeled RA or synthetic retinoids (Fig. 3; Table 2). In competition experiments carried out with [^{3}H] CD367 and unlabeled CD367, K_d values for α, β, and γ receptors were identical to those obtained in saturation experiments (data not shown). Fig. 2 and Table 2 show that the natural ligand, RA displayed a slight RARβ-RARγ selectivity, because the K_d values for these two receptors were 2–3 times lower than the K_d for RARα. Identical data were obtained in saturation experiments using [^{3}H]RA (not shown).

We identified two positional isomers, Am80 and Am580, that displayed a marked selectivity towards RARα (see Fig. 3 and Table 2). The K_d values of these compounds for RARα were 1 order of magnitude lower than the K_d for RARβ and 2 orders of magnitude lower than the K_d for RARγ. In contrast, compounds CD564 and CD495 exhibited a strong RARβ-RARγ selectivity, with a K_d for these receptors 10 times (CD564) and 50 times (CD495) lower than the K_d for RARα.

Functional transactivation assay. HeLa cells were co-transfected with the reporter gene TRE-tk-CAT and each of the RARα, RARβ, and RARγ expression vectors. CAT activity was measured for each retinoid concentration (Fig. 4), and AC_{50} values were determined for all compounds in two to four independent experiments (Table 3). These values can be compared with the K_d values obtained in the binding assays (Table 2). This comparison shows that, except for RA itself, the selectivity in agonist character determined by the functional assay is parallel to the selectivity in affinity determined by the binding assay.

CD367 was found to be an excellent agonist for all three receptors. Am80 and Am580 were found to be good α agonists, with Am580 being, as in the binding assay, both more potent and more selective. CD564 and CD495 were found to be good β-γ agonists. The only discrepancy between the binding assay and the functional transactivation assay was in the case of the natural ligand RA. The slight β-γ selectivity that was found in the binding assay was not found in the functional assay. In agreement with the literature (21), no activation by retinoids of the TRE-tk-CAT reporter gene was observed in the absence of co-transfected expression vectors.

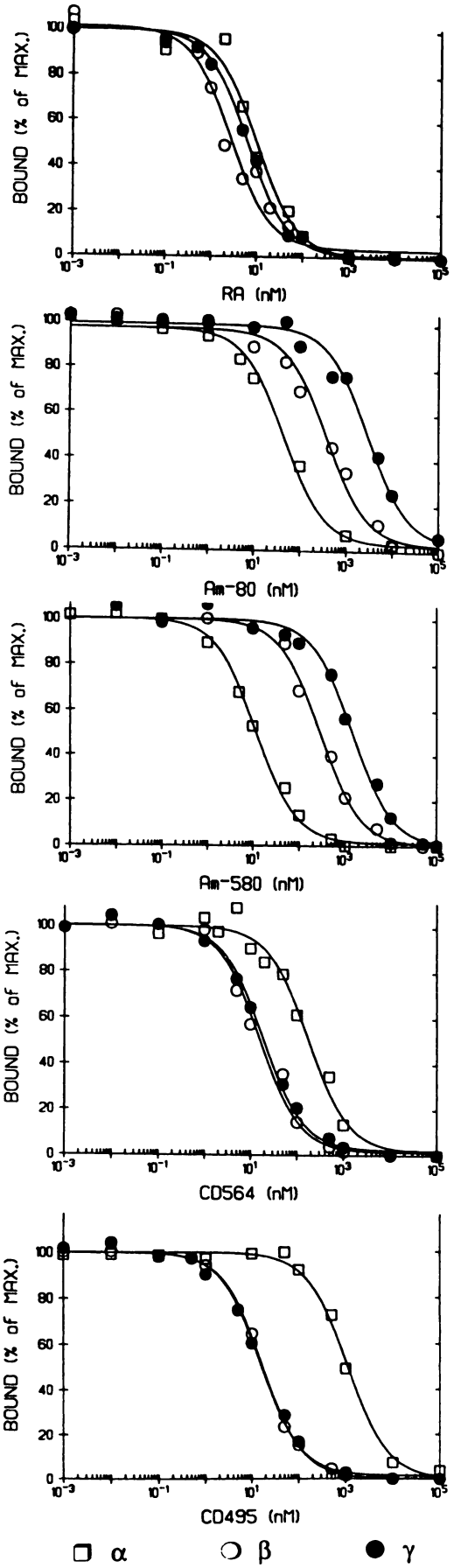

Fig. 3. Competition binding curves of [^{3}H]CD367 with RA, Am80, Am580, CD564, and CD271 on nuclear RARα (□), RARβ (○), and RARγ (●).

TABLE 2

K_d values for RARα, RARβ, and RARγ obtained in saturation experiments with CD367 and in competition experiments with other synthetic retinoids and RA

These values represent the mean of two or three determinations.

Compound	*In vitro* binding, K_d		
	RARα	RARβ	RARγ
		nM	
RA	13	5.5	3.5
CD367	3	4	1.5
Am80	22	280	1720
Am580	6	130	827
CD564	95	6.5	17
CD495	540	8	11

Discussion

In this work, two different assays have been used to evaluate the RARα, RARβ, and RARγ selectivity of retinoids. The first is an *in vitro* binding assay allowing the determination of K_d values for RARα, RARβ, and RARγ produced by transfection with appropriate expression vectors in COS-7 cells. The second is a functional assay for the ability of retinoids to transactivate the reporter gene TRE-tk-CAT through their binding to RARs (α, β, or γ) produced by the cells after co-transfection of appropriate expression vectors. The Scatchard determinations reported here, using extracts from transfected COS-7 cells, give a mean number of binding sites of approximately 20,000 molecules/cell. This figure is close to those reported by Petkovich *et al.* (16) and Gaub *et al.* (41) for transfected COS-1 and HeLa cells. The amount of transfected receptors is then greatly predominant over the amount of endogenous receptors, which can be estimated to be <500 molecules/cell (41). Thus, endogenous receptors should not interfere significantly with the binding determinations and with the functional transactivation assay. Moreover, in HeLa cells, the TRE-tk-CAT reporter is not activated by retinoids and endogenous receptors (see Results and Ref. 21). This is probably due to the fact that TREs have a low affinity for the RARs and require large amounts of receptors to be activated.

The binding assay and the functional assay yield complementary information, because, although both require that the compounds exhibit affinity for the hormone-binding domains of RARα, RARβ, and RARγ, the functional transactivation assay distinguishes between agonist and nonagonist compounds. In principle, discrepancies between the two assays should, therefore, identify partial agonists or antagonists. This did not happen with the synthetic compounds described here, because the binding and functional assays matched perfectly. Although the reference compound CD367 was found to be a nonselective agonist, Am80 and Am580 were found to be selective α agonists, whereas CD564 and CD495 exhibited a RARβ-RARγ selectivity. The RARα selectivity of Am80 was previously suggested by Hashimoto *et al.* (42) in binding assays using RARα and RARβ receptors purified from HL-60 cells. The only discrepancy between the binding and functional assays was in the case of the natural RA ligand itself. This discrepancy may be due to the chemical and metabolic instability of the RA molecule. This is indeed possible, because in the binding assay the incubation is performed at 4° for 2 hr, whereas in the transactivation assay the incubation is done at 37° for 24 hr.

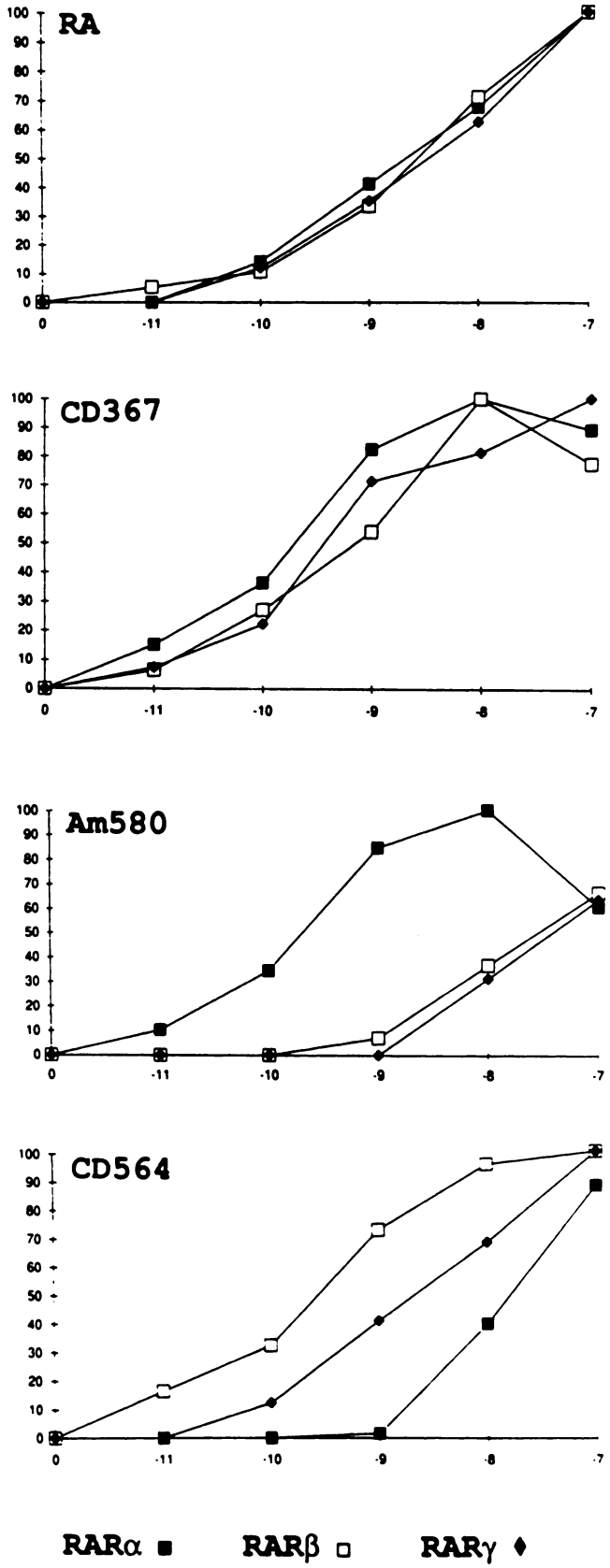

Fig. 4. CAT assays performed with RA, CD367, Am580, and CD564. HeLa cells were co-transfected with TRE-tk-CAT and RARα, RARβ, and RARγ expression vectors. *Abscissa*, log[compound]; *ordinate*, percentage of stimulation.

TABLE 3

AC_{50} values of RA and synthetic retinoids in the functional transactivation assay

These values represent the mean of two to four determinations.

Compound	AC_{50}		
	RARα	RARβ	RARγ
	nM		
RA	2.1	3.6	2.5
CD367	0.2	0.37	0.25
Am80	1.5	6.9	149
Am580	0.36	25	28
CD564	18	0.28	1.2
CD495	2.2	0.18	0.26

This interpretation is supported by the fact that, for the stable synthetic compounds but not for RA, the AC_{50} values were 1 order of magnitude lower than the K_d values. In a previous study in F9 cells (34), where AC_{50} values for induction of plasminogen activator and affinity for endogenous receptors were compared, RA was the only compound found to deviate significantly from the correlation plot.

As already mentioned, selective retinoids should offer invaluable tools for dissecting the biological effects produced by the natural hormone RA and determining, among the elements of the pleiotropic response, whether some effects are mediated by a particular receptor class. Selective retinoids might also open new therapeutic avenues by increasing the benefit to risk ratio. Future studies focused on the structure-activity relationships of a large series of compounds might provide information on the molecular basis of ligand-receptor selectivity.

Our results showing that the members of the RAR family possess differential affinities for synthetic retinoids might bear a physiological significance. Hormonal derivatives of vitamin A, other than RA, may exist *in vivo*, and their effects may be mediated by different classes of RARs and RXRs. This interpretation is supported by the fact that the amino acid sequences of the hormone-binding domains of RARα, RARβ, and RARγ are more conserved from species to species than between each other (22).

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