

# Rational design of RAR-selective ligands revealed by RAR $\beta$ crystal structure

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**The crystal structure of the ligand-binding domain of RAR $\beta$ , a suspect tumour suppressor, reveals important features that distinguish it from the two other RAR isotypes. The most striking difference is an extra cavity allowing RAR $\beta$  to bind more bulky agonists. Accordingly, we identified a ligand that shows RAR $\beta$  selectivity with a 100-fold higher affinity to RAR $\beta$  than to  $\alpha$  or  $\gamma$  isotypes. The structural differences between the three RAR ligand-binding pockets revealed a rationale explaining how a single retinoid can be at the same time an RAR $\alpha$ ,  $\gamma$  antagonist and an RAR $\beta$  agonist. In addition, we demonstrate how to generate an RAR $\beta$  antagonist by gradually modifying the bulkiness of a single substitution. Together, our results provide structural guidelines for the synthesis of RAR $\beta$ -selective agonists and antagonists, allowing for the first time to address pharmacologically the tumour suppressor role of RAR $\beta$  *in vitro* and in animal models.**

Keywords: retinoic acid receptor; 3D structure; ligand-binding domain; ligand design; tumour suppressor

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## INTRODUCTION

The molecular mechanisms by which retinoic acid receptors (RAR $\alpha$ ,  $\beta$ ,  $\gamma$ ) regulate the transcription of target genes in a ligand-dependent manner are in principle understood (for review and references, see Laudet & Gronemeyer, 2002). In the absence of agonists, and in the presence of certain antagonists, RARs, which

form heterodimers with RXRs, are believed to exist in association with target genes in a complex with co-repressors and associated factors, such as histone deacetylases (HDACs), the enzymatic activity of which results in local chromatin condensation and gene silencing. This co-repressor complex is tethered to the RAR–RXR heterodimer through a binding interface that is sensitive to allosteric changes induced by RAR agonists. Consequently, agonists dissociate the co-repressor complex and liberate an overlapping but distinct interaction surface for the binding of coactivator complexes. A major structural feature of the novel (holo-) surface is the contribution of the carboxy-terminally located helix H12, which is re-positioned on the ligand-binding domain (LBD). H12 can adopt several conformations depending on the type of ligand that is bound to the receptor and can be viewed as a positional interpreter of multiple types of agonists and antagonists. Several multiprotein machineries can bind to the holo-surface of RAR–RXR heterodimers; their common feature is the presence of a so-called NR box in the tethering subunit. Coactivator complexes contain histone acetyltransferases (HATs), which allow relieving of the chromatin-mediated silencing induced by HDACs, and ATP-dependent chromatin remodelling machineries prepare the template for the action of the RNA polymerase holo-enzyme. The implication of the individual complexes in specific gene activation, the temporal order of their recruitment, and their promoter and cell specificity are still poorly understood, but the corresponding studies are rapidly advancing (Metivier *et al*, 2003).

The present challenge is to integrate the above structural and molecular biological information into studies on the (patho)physiological relevance of retinoid (and rexinoid) receptor signalling, to provide novel tools for research and therapy. One of the most interesting features of retinoid and rexinoid signalling is its well-documented cancer chemotherapeutic and preventive potential (for recent reviews, see Altucci & Gronemeyer, 2001; Sun & Lotan, 2002). A great number of gene ablation studies (reviewed in Laudet & Gronemeyer, 2002) have, at least to a certain degree, revealed the global impact of retinoid and rexinoid receptors in physiological processes, but can in no way replace receptor

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pharmacology. For example, in the case of the retinoid/rexinoid receptor heterodimer, multiple possibilities of modulating co-repressor and coactivator interaction have been reported (Germain *et al*, 2002); such modulation cannot be mimicked by genetic means. Indeed, the enormous potential of nuclear receptor-based drug design is only insufficiently explored (Gronemeyer *et al*, 2004).

A particularly interesting receptor for cancer research is RAR $\beta$ . The RAR $\beta$  gene is frequently deleted or its expression is epigenetically silenced during cancer progression and RAR $\beta$  re-expression can restore retinoic acid-mediated growth control, suggesting that the anticancer action of retinoids is mediated by RAR $\beta$  (Altucci & Gronemeyer, 2001; Widschwendter *et al*, 2001; Sirchia *et al*, 2002; Sun & Lotan, 2002). Consequently, RAR $\beta$  has been viewed as a tumour suppressor. However, the mechanism underlying its antitumour action has remained elusive. One of the reasons for this is the lack of selective agonists and antagonists. The design of such compounds is extremely difficult, because only one residue in the ligand-binding pocket (LBP) of RAR $\beta$  differs from that of its paralogue RAR $\alpha$ , and two residues differ from RAR $\gamma$ . For this reason, we have set out to define the 3D structure of the RAR $\beta$  LBD. Unexpectedly, the RAR $\beta$  LBP presents features that allow one to develop specific ligands. We report here on a highly RAR $\beta$ -selective agonist and postulate precise guidelines for the rational design of RAR $\alpha$ ,  $\beta$ ,  $\gamma$ -selective agonists and antagonists.

## RESULTS AND DISCUSSION

### Structure determination and refinement

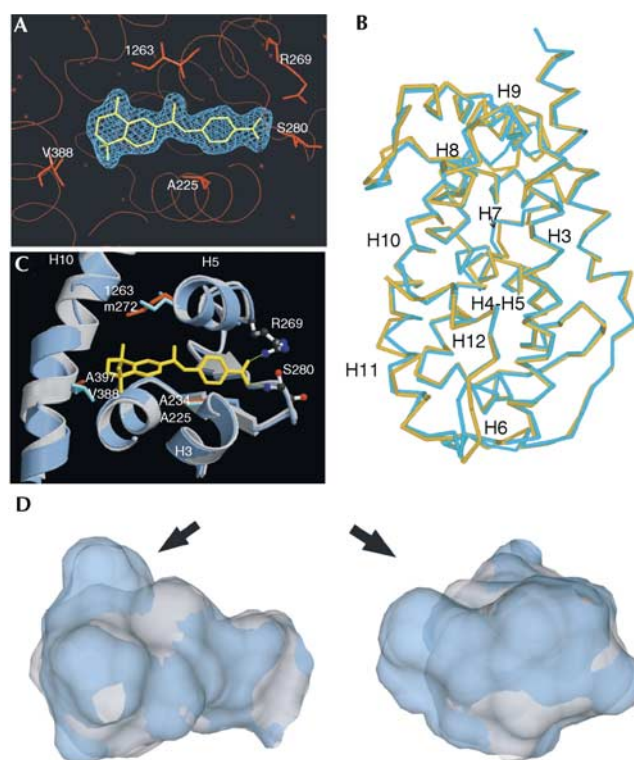
The 3D structure of the RAR $\beta$  LBD–TTNPB complex was solved from a crystal (space group  $P2_12_12_1$ ) that diffracted to 2.1 Å resolution. The RAR $\gamma$  LBD–9-*cis* retinoic acid (9-*cis* RA) complex (Klaholz *et al*, 1998) (PDB, 3lbd) was used as a starting model for molecular replacement. The quality of the electron density map allowed to accurately define the position of the ligand in its binding pocket (Fig 1A). Supplementary Table 1 online summarizes the corresponding crystallographic data. The final model encompasses residues A177–N409; no clear densities were observed for the His tag, the amino-terminal residues 173–176 and for residues 353–355 (loop H9–H10).

### Overall description

The RAR $\beta$  holo-LBD exhibits the canonical antiparallel  $\alpha$ -helical ‘sandwich’ fold of nuclear receptors comprising 12  $\alpha$ -helices and one antiparallel  $\beta$ -sheet; its conformation corresponds to that of agonist complexes (Bourguet *et al*, 2000a). Superposition of TTNPB–RAR $\beta$  and 9-*cis* RA–RAR $\gamma$  LBD (Renaud *et al*, 1995; PDB, 3lbd) complexes using the LSQ options from O revealed a generally high degree of structural overlap (r.m.s. deviation, 0.75 Å; Fig 1B). Although the observed shift of RAR $\gamma$  helices H10 and H11 towards the surface may be because of the bulkier volume of TTNPB compared with 9-*cis* RA, possibly stabilized by crystal packing contacts not observed in the 9-*cis* RA–RAR $\gamma$  LBD crystal, we do not exclude a RAR $\beta$ -specific conformation of this region.

### Ligand-binding pocket

RAR $\beta$  accommodates TTNPB in the canonical cavity between the  $\beta$ -turn and H11 in one, and H5 and H3/H12 in the other direction (Fig 1C). The hydrogen bond network anchoring the ligand carboxylate is identical to that formed in RAR $\gamma$  (Renaud *et al*,



**Fig 1** | Crystal structure of the RAR $\beta$  LBD–TTNPB complex reveals an additional cavity in the RAR $\beta$  LBP. (A) Electron density map of TTNPB in its pocket.  $2F_o - F_c$  map at 2.1 Å resolution contoured at  $1\sigma$ . The three isotype-specific residues (A225, I263 and V388) and residues anchoring the carboxylate (R269 and S280) are indicated. Illustration by PYMOL. (B) Superposition of the holo-RAR $\beta$ –TTNPB (yellow) and RAR $\gamma$ –9-*cis* RA (blue) LBDs. Illustration by SETOR. (C) Superposition of TTNPB–RAR $\beta$  (blue) and 9-*cis* RA–RAR $\gamma$  (grey) LBDs. The isotope-specific residues are shown in cyan (RAR $\beta$ ) and orange (RAR $\gamma$ ) and TTNPB in yellow. The carboxylate anchoring residues are illustrated as ball-and-sticks. H bonds are represented as dashed lines. The figure was prepared by MOLSCRIPT and RASTER3D. (D) Superimposition of RAR $\beta$  (blue) and RAR $\gamma$  (grey) LBPs. The arrow points to the additional cavity in RAR $\beta$ .

1995; Klaholz *et al*, 2000b). Briefly, one of the carboxylate oxygen atoms interacts with N $\epsilon$  of RAR $\beta$  R269 (3.08 Å) of the loop connecting H5 to the N-terminal  $\beta$ -strand, whereas the other oxygen is involved in a hydrogen bond network with N $\alpha$  of S280 (2.69 Å) of the  $\beta$ -turn and one invariant water molecule (2.71 Å; Fig 1C; supplementary Fig S1 online). All TTNPB carbon atoms establish van der Waals contacts with residues lining the LBP (supplementary Fig S1 online). Compared with 9-*cis* RA-bound RAR $\gamma$ , no unexpected interactions were noted for TTNPB binding to RAR $\beta$ , in keeping with the fact that only two residues differ between the two LBPs (Renaud *et al*, 1995; Gehin *et al*, 1999; Bourguet *et al*, 2000b).

Superposition with RAR $\gamma$  reveals that the LBP of RAR $\beta$  is significantly larger (Fig 1D). Using VOIDOO, a volume of 503.5 Å<sup>3</sup> was determined, whereas that in the 9-*cis* RA–RAR $\gamma$  complex is 429.4 Å<sup>3</sup>. This difference is not a mere conformational adaptation to the ligand but reflects a different architecture of the

two LBPs, because (i) the maximal observed volume for the RAR $\gamma$  LBP occupied by various ligands was 457.2 Å<sup>3</sup> (Klaholz *et al*, 2000b), thus >9% smaller than the RAR $\beta$  cavity, and (ii) the greater volume of the RAR $\beta$  pocket cannot be explained by steric repulsions or crystal packing effects. Instead, different conformations of one of the two divergent LBP residues account for the different RAR $\beta$  and RAR $\gamma$  LBPs. In RAR $\beta$ , the I<sub>263</sub> side chain is positioned such that an additional cavity is generated between H5 and H10 (Fig 1C). This cavity does not exist in RAR $\gamma$  as M<sub>272</sub> points inside the LBP (Klaholz *et al*, 1998). These structural features suggest that ligands occupying the additional space (arrow in Fig 1D) may acquire RAR $\beta$  selectivity.

### Structural basis of an RAR $\beta$ agonist–RAR $\gamma$ antagonist

The characterization of retinoids has revealed not only pan- and RAR-selective agonists and antagonists, but also a class of compounds that can act as agonists with one and antagonists with another RAR, such as BMS453 (Chen *et al*, 1995). The structural basis of this phenomenon has remained elusive. Competition curves derived from challenging 3 nM TTNPB with increasing BMS453 concentrations reveal that BMS453 is a potent antagonist of TTNPB-induced transcription for Gal-RAR $\alpha$  and Gal-RAR $\gamma$ , whereas it acts as a ‘mixed’ agonist/antagonist (i.e. a weaker agonist than TTNPB) for RAR $\beta$  (Fig 2A,B; structures are given in Fig 4A).

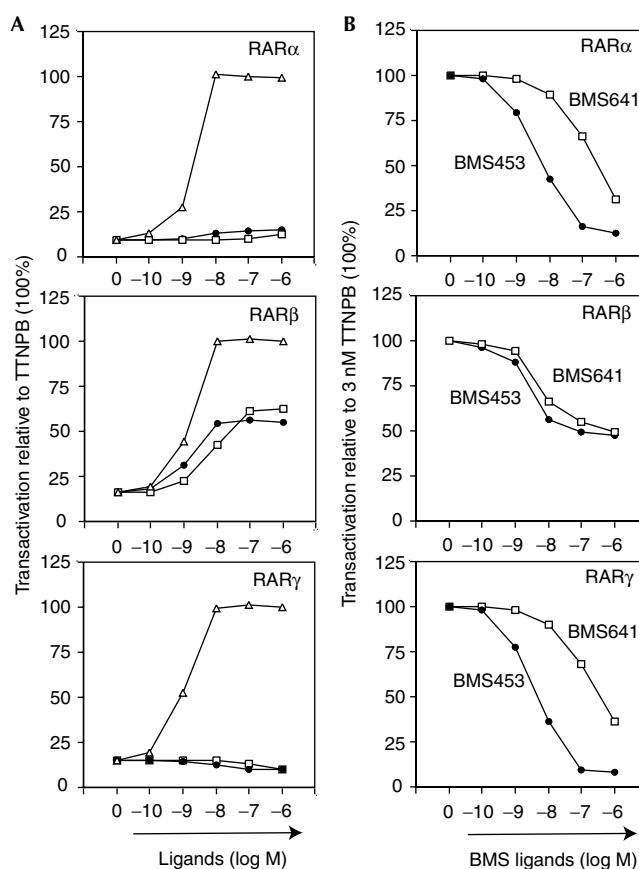
To provide a structural rationale explaining the different transcriptional activities of BMS453 on binding to the three RARs, we performed docking experiments and compared the corresponding fitness scores for the RAR $\beta$  and RAR $\gamma$  LBPs using GOLD. The validation of the GOLD energy function for retinoid docking is described in the supplementary information online.

Docking of BMS453 into the RAR $\beta$  LBP gave similar solutions, with favourable scores; the ligand was completely buried in the LBP with the phenyl oriented towards H5 (Fig 3A shows one solution of VOIDOO). In contrast, any of the proposed solutions for hRAR $\gamma$  had low fitness scores, indicating substantial docking problems. Indeed, inspection of the two predicted types of ligand conformations (Fig 3B, red and green) revealed steric clashes with I<sub>412</sub> of H12 (in the agonist conformation) and A<sub>234</sub> of H3 for the ‘green’ solution, and with M<sub>272</sub> of H5 for the ‘red’ solution. These distinct docking results reflect the smaller size of the RAR $\gamma$  LBP and the additional cavity in the RAR $\beta$  LBP, which accommodates the bulky ‘head’ of the molecule with its phenyl moiety.

Removing H12 from the RAR $\gamma$  LBP structure allowed to manually dock BMS453 readily in the LBP, thus suggesting that this ligand could act as an RAR $\gamma$  antagonist as its binding is incompatible with an agonist conformation. Indeed, transactivation assays (Fig 2B, bottom) confirmed this notion. Together, the above considerations provide a structural rationale accounting for the RAR $\beta$  agonist–RAR $\gamma$  antagonist activities of BMS453.

### Converting an RAR $\beta$ agonist into an antagonist

Inspired by the structural analysis of the agonist–antagonist switch of BMS453, we predicted that increasing the bulkiness of the hydrophobic group of BMS453-like molecules would generate RAR $\beta$  ligands that sterically interfere with the agonist positioning of H12 and thus turn into RAR $\beta$  antagonists. Indeed, studying the agonist–antagonist characteristics of a series of 8'' substituted (*E*)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene-2-yl)vinyl]-benzoic acid derivatives (Fig 4A) revealed that a

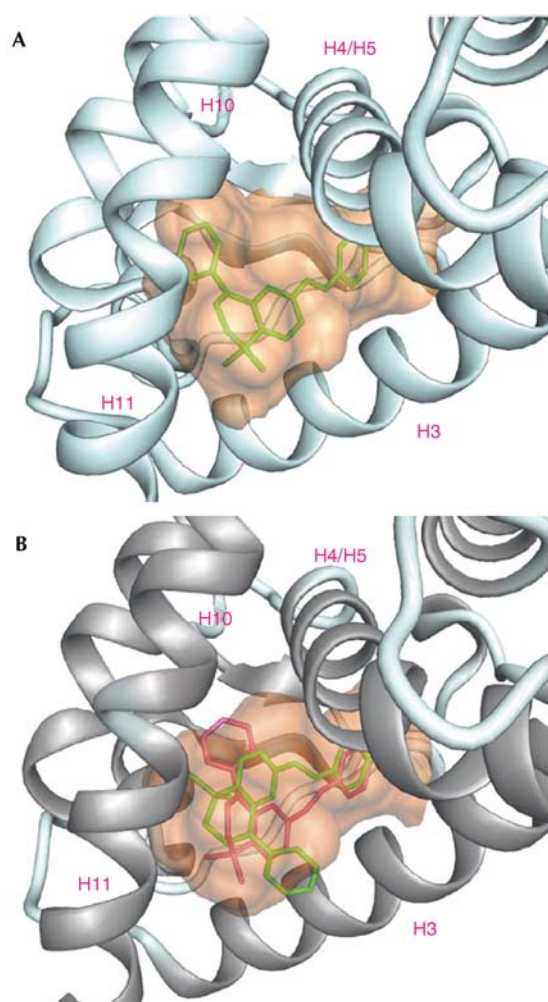


**Fig 2** | Agonist–antagonist potential and RAR $\beta$  selectivity of BMS453 and BMS641. (A) HeLa cells were co-transfected with reporter (17m)5x-G-luc and Gal-RAR $\alpha$ , Gal-RAR $\beta$  or Gal-RAR $\gamma$ , as indicated. Cells were incubated with increasing concentrations of TTNPB (open triangles), BMS453 (closed circles) or BMS641 (open squares). (B) Transient transactivation assays as in (A) to assess antagonistic activities of BMS453 (closed circles) or BMS641 (open squares). The reporter was activated with 3 nM TTNPB (100%) and increasing concentrations of the synthetic retinoids were added.

(*Z*)-3,3-dimethylbut-2-en-1-yl substitution yielded a full RAR $\beta$  agonist (BMS987 in Fig 4B) virtually indistinguishable from TTNPB. BMS453 has a bulkier phenyl moiety in 8'' position and its weaker activity suggests that the phenyl may cause some weak interference with H12 positioning and coactivator recruitment. Increasing the bulkiness at 8'' position by introducing a *p*-tolyl group (BMS701) further reduced the agonist activity of the ligand, and a *p*-biphenyl substitution yielded a high-affinity antagonist (BMS009 in Fig 4). All ligands of this series show similar affinity for RAR $\beta$  ( $K_d$ , 2–9 nM) excluding that different affinities could account for distinct transcriptional activities.

### Identification of an RAR $\beta$ -selective agonist

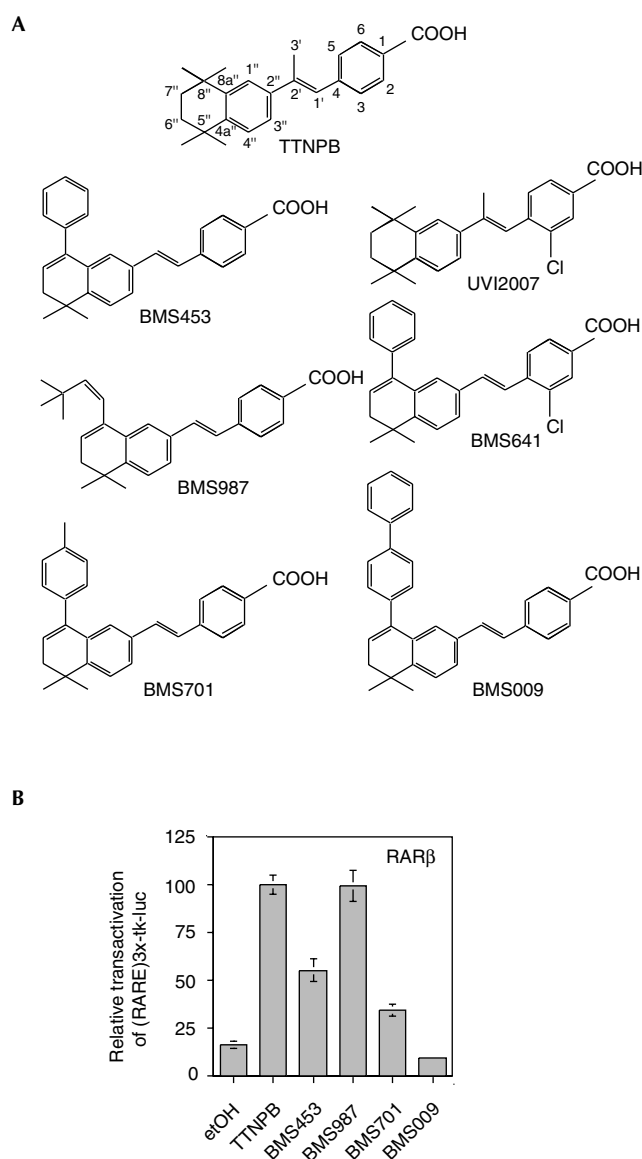
By screening a panel of synthetic retinoids with an *in vivo* reporter cell system (Chen *et al*, 1995), we identified BMS641, a retinoid that exclusively activated RAR $\beta$  in reporter cells. Limited proteolysis with trypsin (Fig 5A, middle panel) revealed BMS641-dependent protection of RAR $\beta$  similar to that seen with



**Fig 3** | Docking of BMS453 into the RAR $\beta$  (A) and RAR $\gamma$  (B) LBP. The ligand cavities were calculated by VOIDOO and MSMS using a probe radius of 1.4 Å. DINO was used for illustration. (A) BMS453 in green is perfectly buried within the RAR $\beta$  LBP with its phenyl ring oriented towards H12. (B) The two predicted orientations (green and red) could not be accommodated in the RAR $\gamma$  cavity.

the panRAR agonist TTNPB (left panel), but no protection of RAR $\alpha$  or RAR $\gamma$ . Indeed, direct binding assays revealed a higher affinity for RAR $\beta$  ( $K_d$ , 2.5 nM) that was 100 times higher than that for RAR $\alpha$  ( $K_d$ , 225 nM) or RAR $\gamma$  ( $K_d$ , 223 nM). In contrast, BMS453 induced a digestion pattern very similar to that of TTNPB, indicating that BMS453 can bind to all three RARs with similar affinities. Interestingly, BMS641 is a 3-chloro derivative of BMS453 implying that a halogen in position 3 is a crucial determinant for generating RAR $\beta$  binding selectivity.

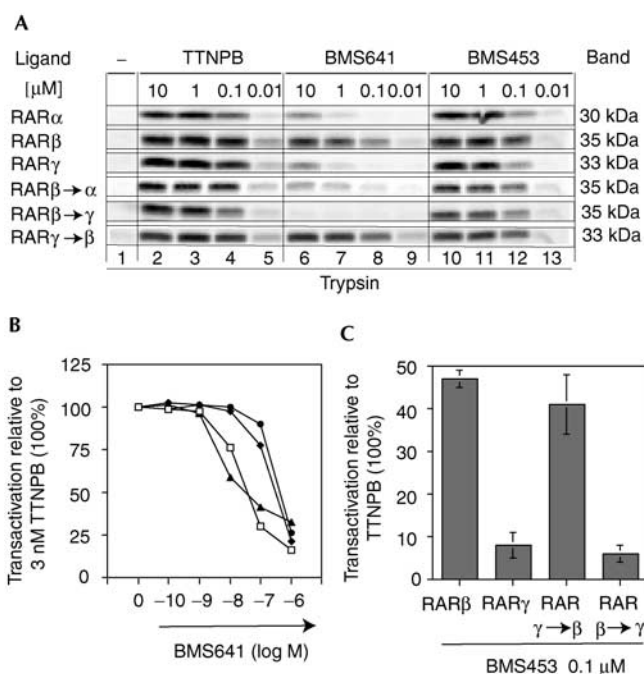
Transient transactivation (Fig 2) reflected ligand binding. BMS641 did not antagonize TTNPB-induced transcription for RAR $\alpha$  and RAR $\gamma$ , in keeping with its low affinity for these receptors. In contrast, a 'mixed' agonistic/antagonistic activity was seen for RAR $\beta$ , as competition curves plateaued at about 50% of the TTNPB-induced activity. Virtually identical inhibition was obtained with BMS453, in keeping with similar relative binding and agonistic activities of BMS453 and BMS641 for RAR $\beta$ .



**Fig 4** | RAR $\beta$  agonist potential of a series of 8''-substituted TTNPB-like retinoids. (A) Chemical structures of the retinoids used in this study. (B) Transactivation assays. HeLa cells were co-transfected with the reporter (RARE)3x-tk-luc and RAR $\beta$  in the presence of the indicated BMS compounds at 0.1  $\mu$ M.

### Three residues dictate RAR $\beta$ selectivity

We previously demonstrated that swapping of three divergent residues in the LBPs of RARs suffices to modulate isotype-selective binding (Gehin *et al*, 1999). To assess whether these residues are also important for RAR $\beta$ -selective binding of BMS641, partial proteolysis maps were established (Fig 5A). Indeed, converting the single divergent residue of RAR $\beta$  into that of RAR $\alpha$  (mutant RAR $\beta$   $\rightarrow$   $\alpha$ ; RAR $\beta_{A225S}$ ) or two divergent residues into those of RAR $\gamma$  (mutant RAR $\beta$   $\rightarrow$   $\gamma$ ; RAR $\beta_{(I263M, V388A)}$ ) resulted in a loss of BMS641-dependent protection, whereas a gain of protection was observed with RAR $\gamma$  if its LBP was changed to that of RAR $\beta$  (mutant RAR $\gamma$   $\rightarrow$   $\beta$ ; RAR $\gamma_{(M272L, A397V)}$ ). Importantly, identical

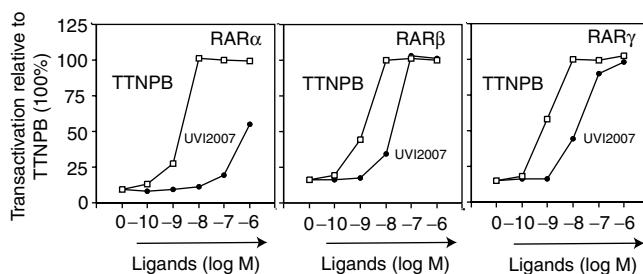


**Fig 5** | The three divergent LBP residues determine the RAR $\beta$  selectivity of BMS641 and the isotype-dependent potential of BMS453. (A) Partial proteolysis maps of *in vitro*-translated RARs in the presence or absence of increasing concentrations of either TTNPB, BMS453 or BMS641, as indicated. (B) Dose-response curves to assess the binding affinity of BMS641 relative to TTNPB in RAR pocket mutants. HeLa cells were co-transfected with (RARE)3x-tk-luc and either RAR $\beta$  (closed triangles), RAR $\gamma$  (open circles), RAR $\gamma \rightarrow \beta$  (RAR $\gamma$ (M<sub>272</sub>I, A<sub>397</sub>V); open squares) or RAR $\beta \rightarrow \gamma$  (RAR $\beta$ (I<sub>263</sub>M, V<sub>388</sub>A); closed diamonds) and reporter gene transcription was induced with 3 nM TTNPB (100%). (C) BMS453-induced luciferase activity in HeLa cells co-transfected with (RARE)3x-tk-luc and the indicated receptors.

maps were obtained with TTNPB or BMS453 (Fig 5A). Transactivation experiments supported these results (Fig 5B) and showed that these residues also determine the agonistic/antagonistic profile of ligands with this chemotype (Fig 5C). Indeed, in the context of the mutant RAR $\gamma \rightarrow \beta$  BMS453 acts as a partial agonist (~40% of TTNPB), but when the LBD is converted into that of RAR $\gamma$  (mutant RAR $\beta \rightarrow \gamma$ ) BMS453 shows a strong antagonistic activity as with wild-type RAR $\gamma$  (less than 5% of the TTNPB activity). These results underscore the importance of the divergent residues and suggest that a 3-chloro substitution in TTNPB-like retinoids may sense the RAR $\beta$  pattern.

### A 3-chloro substitution is RAR $\alpha$ discriminatory

To study whether a 3-chloro substitution on its own would generate RAR $\beta$  selectivity, we introduced it into the pan-agonist TTNPB, generating UVI2007 (Figure 4A; see supplementary section online for synthesis). Transcriptional (Fig 6) and proteolysis (not shown) assays demonstrated that UVI2007 shows a lower relative binding affinity to all three RAR isotypes. Notably, the decrease in the affinity is much more pronounced for RAR $\alpha$  than for RAR $\beta$  or  $\gamma$ , revealing an RAR $\alpha$  discriminatory effect of the



**Fig 6** | A 3-chloro substitution discriminates RAR $\alpha$  from RAR $\beta/\gamma$  binding. HeLa cells were transfected as in Fig 2A. Dose-response curves for TTNPB (open squares) or UVI 2007 (closed circles).

3-chloro group. This is most likely because of a steric interference originating from RAR $\alpha$ S<sub>232</sub>, which is bulkier than RAR $\beta$ A<sub>225</sub>. In contrast to BMS641, no significant difference was seen for UVI2007 binding to RAR $\beta$  and RAR $\gamma$  (Fig 6), indicating that on its own a chlorine in position 3 is insufficient to discriminate between these isotypes.

### Structural features contributing to RAR $\beta$ selectivity

From this and previous studies (reviewed in Bourguet *et al*, 2000a), the following structural features can be deduced for the synthesis of RAR isotype-selective retinoids: (i) the three divergent residues and the different shape of the RAR $\beta$  LBP reported here are the most important discriminatory elements; (ii) existing isotype-selective ligands dissociate mostly RAR $\alpha$  from RAR $\beta/\gamma$  by exploiting the presence of RAR $\alpha$ S<sub>232</sub>, which can establish hydrogen bonds with suitable ligands; this was predicted for ligands harbouring an amino group, such as Am580 or BMS753 (Gehin *et al*, 1999); (iii) to separate RAR $\beta$  from RAR $\alpha$  binding, a 3-chloro substitution that appears to create steric hindrance within the RAR $\alpha$  pocket can be introduced in TTNPB-like retinoids; (iv) to separate RAR $\beta$  from RAR $\gamma$  binding, two structural aspects can be exploited, both of which are a consequence of the replacement of RAR $\beta$ I<sub>263</sub> by RAR $\gamma$ M<sub>272</sub>. First, the side-chain orientation of I<sub>263</sub> opens a cavity that is closed in RAR $\gamma$ ; ligands that require such a cavity to accommodate a bulky substituent acquire  $\beta$ -selectivity. Our functional analyses have shown that a 3-chloro (in UVI2007) or an 8''-phenyl (in BMS453) on its own cannot discriminate between RAR $\beta$  and RAR $\gamma$ ; however, the combination of both substitutions (in BMS641) results, despite an overall lower affinity, in the acquisition of  $\beta$ -selective binding that originates from a marked loss of RAR $\gamma$  affinity. This is highly suggestive of a structural model in which the 3-chloro substitution positions the ligand in the RAR $\gamma$  pocket such that the 8''-phenyl clashes with M<sub>272</sub> (but not the corresponding I<sub>263</sub> of RAR $\beta$ ). Second, the presence of M<sub>272</sub> allows establishing hydrogen bonds to a suitable ligand, for example, BMS270394 (Klaholz *et al*, 2000a), to augment  $\gamma$  affinity and selectivity; (v) in all cases, antagonists are characterized by the presence of substitutions that interfere with the holo-conformation of H12.

### SPECULATION

The novel structure of the RAR $\beta$  LBD allows, for the first time to our knowledge, the establishment of a comprehensive set of guidelines for the rational drug design of RAR-selective retinoids.

The availability of agonists and antagonists selective for each of the RARs will allow one to dissect the various activities exerted by the three RARs, which are the basis for the well-established cancer therapeutic and cancer preventive activity of retinoids (Altucci & Gronemeyer, 2001; Sun & Lotan, 2002), assess the role of the suspect tumour suppressor RAR $\beta$  and will aid in generating novel retinoids with reduced side effects.

## METHODS

**Software.** The software used in this study is described in the supplementary section.

**Ligands and plasmids.** For ligands, see the supplementary section online and supplementary Table 2 online. pSG5-based RAR and GAL-RAR expression vectors and reporter genes were described previously (Nagpal et al, 1993; Chen et al, 1995; Gehin et al, 1999). For docking experiments, ligands with all hydrogen atoms were built with QUANTA-CHARMM.

**Protein purification, crystallization and data collection.** The His-tagged hRAR $\beta$  LBD (173–409) was purified to 98% homogeneity and crystallized as described in the supplementary section online. X-ray diffraction data were collected (Synchrotron Light Source, Zürich, Switzerland) and processed with DENZO and SCALEPACK from the HKL2000 package.

**Structure refinement.** Structure determination and refinement was carried out with CNS solve package using the hRAR $\gamma$  LBD complex (PDB ID, 3LBD) as a search model. Data between 20 and 2.1 Å resolution were included in the refinement process (supplementary section online and supplementary Table 1 online).

**Cell culture and transient transfections.** HeLa cells, cultured in Dulbecco's modified Eagle's medium–5% fetal calf serum, were transfected as described (Vivat et al, 1997).

**Limited proteolytic digestion.** For limited proteolytic digestions (Vivat et al, 1997), *in vitro*-made <sup>35</sup>S-labelled human RARs (TNT kit, Promega) were used. Briefly, after incubating on ice for 1 h with ligand, receptor proteins were digested at 25 °C for 10 min with 100 µg/ml (RAR $\alpha$ , RAR $\beta$ ) or 50 µg/ml (RAR $\gamma$ ) trypsin.

**Supplementary information** is available at *EMBO reports* online (<http://www.emboports.org>).

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