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Retinoic acid-induced developmental defects are mediated by RARβ/RXR heterodimers in the pharyngeal endoderm

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SUMMARY

Fusion and hypoplasia of the first two branchial arches, a defect typically observed in retinoic acid (RA) embryopathy, is generated in cultured mouse embryos upon treatment with BMS453, a synthetic compound that exhibits retinoic acid receptor β (RARβ) agonistic properties in transfected cells. By contrast, no branchial arch defects are observed following treatment with synthetic retinoids that exhibit RARα or RARγ agonistic properties. The BMS453-induced branchial arch defects are mediated through RAR activation, as they are similar to those generated by a selective pan-RAR agonist, are prevented by a selective pan-RAR antagonist and cannot be mimicked by exposure to a pan-RXR agonist alone. They are enhanced in the presence of a pan-RXR agonist, and cannot be generated in *Rarb*-null embryos. Furthermore, they are accompanied, in the morphologically altered region, by ectopic expression of *Rarb* and of several other direct RA target genes. Therefore, craniofacial abnormalities characteristic of the RA embryopathy are mediated through ectopic activation of RARβ/RXR heterodimers, in which the ligand-dependent activity of RXR is subordinated to that of RARβ. Endodermal cells lining the first two branchial arches respond to treatment with the RARβ agonist, in contrast to neural crest cells and ectoderm, which suggests that a faulty endodermal regionalization is directly responsible for RA-induced branchial arch dysmorphologies. Additionally, we provide the first in vivo evidence that the synthetic RARβ agonist BMS453 exhibits an antagonistic activity on the two other RAR isotypes.

Key words: Retinoic acid embryopathy, Synthetic retinoids, Nuclear receptors, Embryo cultures, Endoderm, Branchial arches, Pharyngeal pouches, Mouse, Synergy, Agonists, Antagonists, Teratogenicity

INTRODUCTION

Retinoic acids (RAs), the active metabolites of vitamin A, exert their pleiotropic effects through binding to two families of nuclear receptors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs), each family comprising three isotypes (α, β and γ). Either all-trans or 9-cis RA activates RARs, whereas only 9-cis RA activates RXRs. RARs and RXRs function as ligand-dependent transregulators by binding, in the form of RAR/RXR heterodimers, to RA response elements (RARE) located in the regulatory regions of target genes (reviewed by Chambon, 1996; Laudet and Gronemeyer, 2002). RXRs can also act as homodimers (at least in vitro), and as heterodimerization partners for numerous nuclear receptors in addition to RARs: thyroid hormone receptors, vitamin D3 receptor, peroxisome proliferator activated receptors and several orphan receptors (reviewed by Mangelsdorf and Evans, 1995; Chambon, 1996). A systematic germline knockout approach of RARs and RXRs in the mouse, performed over the past 10 years, has demonstrated that: (1) RARs and RXRs are required to mediate the developmental effects of RA; (2) functional redundancies exist among the three RARs and among the three RXRs; (3) RAR/RXRα heterodimers are the main functional units that transduce the retinoid signal during development; and (4) transcriptional activation of both partners in these heterodimers is often required to activate target genes and to mediate the physiological effects of RA during morphogenesis and organogenesis (reviewed by Kastner et al., 1995; Mark et al., 1999; Kastner et al., 1997; Mascrez et al., 1998; Mascrez et al., 2001).

RA is indispensable for early morphogenesis and for organogenesis as these are dramatically disturbed when the physiological level of RA is lowered (Niederreither et al., 1999) and when RAR/RXR-mediated signalling pathways are abrogated by genetic (see above) or pharmacological approaches (Wendling et al., 2000; Wendling et al., 2001; Schneider et al., 2001). However, RA is also a potent teratogen that, at pharmacological concentrations, can induce congenital defects in all vertebrate species as well as in certain invertebrates (Soprano and Soprano, 1995; Collins and Mao, 1999; Escriva et al., 2002). In humans, oral intake of Accutane (13-cis RA) during gastrulation and early organogenesis (gestational weeks 2-5) results in a spectrum of malformations referred to as retinoic acid embryopathy (RAE) (Lammer et al., 1985).

An external ear malformation is the most frequent defect

observed in human RAE and in mammalian models of RAE (Lammer et al., 1985; Webster et al., 1986; Wei et al., 1999). The external ear and the other craniofacial components commonly deficient in RAE syndromes, the mandible and the middle ear (Lammer et al., 1985; Coberly et al., 1996; Mallo, 1997), originate from the first two branchial arches (BAs), the first branchial cleft and the first pharyngeal pouch (Larsen, 1993). BAs are transient bulges of the embryonic surface that flank the oral and pharyngeal cavities, and develop in a cranial to caudal sequence. Each consists of a mesenchymal core covered externally by ectoderm and lined internally by endoderm. Grooves of the BA ectoderm (branchial clefts) and evagination of the pharyngeal endoderm (pharyngeal pouches) separate the BAs from one another. The mesenchyme of the first two BAs is largely made of neural crest cells (NCC) that have emigrated from the caudal midbrain and the anterior hindbrain (Lumsden et al., 1991; Serbedzija et al., 1992). Maternal exposure of mouse embryos to RA at embryonic day (E) 8.0 results in fusion and hypoplasia of the first two BAs. It has been assumed that such BA defects could account for alterations of the external ear and mandible displayed at birth by RA-exposed embryos, and that neural crest is the primary target tissue of RA-induced teratogenesis in the BA region (Goulding and Pratt, 1986; Webster et al., 1986; Pratt et al., 1987; Lee et al., 1995; Wei et al., 1999).

The aim of the present study was to gain insights into the cellular and molecular mechanisms underlying the RA-induced BA defects and more specifically to: (1) evaluate the relative contributions of RARs, RXRs and individual RAR isotypes to the generation of these defects; and (2) characterize the pathogenetic events underlying RA-induced teratogenesis. To this end, we have analyzed the effects of various synthetic agonistic and antagonistic retinoids on BA formation in wild-type and *Rarb*-null embryos in culture.

MATERIALS AND METHODS

Retinoids, embryo cultures and mouse lines

The synthetic agonist retinoids, selective for RARα (BMS753), RARβ (BMS453), and RARγ (BMS961), the panRXR agonist (BMS649, identical to SR11237) (Gendimenico et al., 1994), and the panRAR antagonist BMS493 were gifts from Bristol-Myers Squibb (Wallingford, CT). Their selectivity was assessed by transactivation assays, direct binding assays (Chen et al., 1995; Gehin et al., 1999; Germain et al., 2002) and, in the case of BMS493, an in vivo assay (Wendling et al., 2000; Wendling et al., 2001). TTNPB (Strickland et al., 1983) was purchased from TEBU (France).

Embryos collected at E8.0 (two- to four-somite stage) were cultured, as described previously (Copp and Cockroft, 1990), for 12, 24, 30 or 48 hours. Retinoids were diluted in ethanol and added to the culture medium to give a final concentration of 0.1% (vol/vol). In control cultures, the ethanol vehicle was added at the same final dilution. Mouse lines carrying the *Rarb2-lacZ* as well as the *Rare-hsp68-lacZ* RA-reporter transgenes have been described previously (Mendelsohn et al., 1991; Rossant et al., 1991). *Rarb*-null embryos were generated from intercrosses between $Rarb^{+/-}$ mice and were genotyped at the end of the culture, as described (Ghyselinck et al., 1997).

External morphology, histology, ink injection and in situ hybridization

Cultured embryos were fixed in Bouin's fluid for 5 hours, rapidly rinsed in 70% ethanol and then in phosphate-buffered saline (PBS), stained for 3 minutes in Acridine Orange (10 μg/ml in PBS, Sigma) according to Zucker et al. (Zucker et al., 1995), and photographed. Embryos were then post-fixed in Bouin's fluid for 16 hours, and processed for histology according to standard procedures. Staining for β-galactosidase activity was carried out as described (Mendelsohn et al., 1991). Embryos were post-fixed in 4% buffered paraformaldehyde (PFA) for 16 hours at 4°C and processed for histology. To visualize the aortic arches, embryos cultured for 48 hours were injected with Pelikan Ink (number 17) via the yolk sac vasculature using a 1 mm capillary tube, and then fixed in 4% PFA for 16 hours and cleared according to Waldo et al. (Waldo et al., 1990).

For in situ hybridization, cultured embryos were fixed by 4% PFA in PBS (1 hour; 4°C). The digoxigenin-labelled antisense riboprobes were synthesized by T7 polymerase using *Rarb* (all isoforms) (Ruberte et al., 1991), *Hoxa1* (Duboule and Dollé, 1989), *Hoxb1* (Frohman et al., 1990) and *Pax1* (Deutsch et al., 1998) cDNA as templates. Whole-mount in situ RNA hybridization was carried out as described (Décimo et al., 1995), except that 100 mM maleic acid, 150 mM NaCl, 0.1% Tween 20 was used for the washes. In situ hybridization was performed according to Myat et al. (Myat et al., 1996) with the following modifications: 10 μm paraffin wax-embedded sections were rehydrated in water and 120 μl of the heat inactivated RNA probe (diluted 1/100 in hybridization buffer) was applied on each slide. The anti-DIG antibody (Roche, Germany) was diluted 1/2500 in blocking solution. Sections were incubated in NBT/BCIP (the two substrates for alkaline phosphatase; Boehringer Mannheim, Germany) for 48 hours, with one change of the staining solution after 24 hours.

RESULTS

Embryos were collected at E8.0 (two- to four-somites stage), i.e. about 12 hours prior to the appearance of the mandibular component of the first BA (Kaufman, 1992), and cultured for 12, 24, 30 or 48 hours. In these cultures, the second and third BAs were readily identifiable after 30 and 48 hours, respectively, and the morphology of control embryos exposed to ethanol (i.e. the retinoid vehicle) for 48 hours was similar to that of E9.5 embryos developing in vivo (see, for example, Fig. 1A, Fig. 2A and Fig. 4A-C) (Wendling et al., 2000).

The panRAR-selective agonist TTNPB and the RARβ-selective agonist BMS453 induce identical branchial arch defects

To determine whether RARs or RXRs are involved in fusion and hypoplasia of the first two BAs (termed hereafter 1-2BAFH) induced by excess RA (Goulding and Pratt, 1986; Webster et al., 1986), wild-type embryos were cultured in the presence of either the panRAR-selective agonistic retinoid TTNPB or the panRXR-selective agonistic rexinoid BMS649 (SR11237). Treatment with 5.10^{-9} M TTNPB (n=10 embryos) induced malformations restricted to the first two BAs, which were consistently smaller than in control embryos and fused (compare B1 and B2 in Fig. 1A with B1-2 in Fig. 1B) instead of separated by the first branchial cleft (arrowhead in Fig. 1A, compare with Fig. 1B). By contrast, treatment with the rexinoid BMS649 did not induce morphological alterations, even at a concentration of 10^{-6} M (n=18; data not shown). A RAR-selective retinoid is therefore sufficient to induce the teratogenic effects of RA on BA development, whereas a RXR-selective retinoid (i.e. a rexinoid) has no effect on its own.

Wild-type embryos (n=95) cultured in the presence of

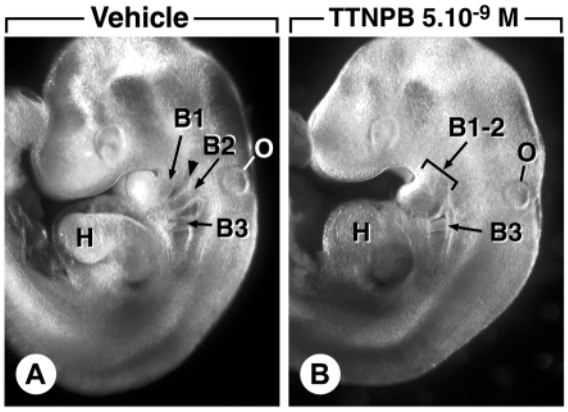

Fig. 1. Effects of the panRAR-selective agonist TTNPB on branchial arch development. Morphology of vehicle-treated (A) and TTNPB-treated (B) wild-type embryos after 48 hours in culture. B1-B3, branchial arches 1 to 3, respectively; B1-2, fused first and second branchial arches; H, heart; O, otocyst. Arrowhead indicates the first branchial cleft.

10^{-7} M RARβ-selective agonist BMS453 reproducibly displayed an external defect consisting of a 1-2BAFH (compare B1 and B2 in Fig. 2A with B1-2 in Fig. 2B) with a small or absent first branchial cleft (arrowhead in Fig. 2A, compare with Fig. 2B). Variable degrees of fusion between the first two BA arteries (i.e. the first and second aortic arches; A1 and A2 in Fig. 2C) were seen following injection of ink into the embryonic vasculature (A1-2 in Fig. 2D), and on serial histological sections (compare A1 and A2 in Fig. 2E with A1-2 in Fig. 2F). The first and second pharyngeal pouches were markedly hypoplastic on flat mounts of isolated pharyngeal endoderm (P1 and P2, compare Fig. 3G,I,K with Fig. 3H,J,L). In situ hybridization with a *Pax1* probe was used to investigate pharyngeal pouch development. Similar to what is observed during normal embryogenesis, vehicle-treated embryos cultured for 12, 24 and 48 hours exhibited high levels of *Pax1* expression in the walls of the forming first and second pouches, but not in their most lateral region, which is close to the surface ectoderm (P1 and P2, Fig. 3A,C,E,G,I,K) (Müller et al., 1996; Dupé et al., 1999). In BMS453-treated embryos, *Pax1* was weakly expressed in the first and second pouches, and sometimes expression was undetectable in the first pouch (P1 and P2, Fig. 3B,D,F,H,J,L). Moreover, these two pouches often appeared to have fused together in embryos cultured for 48 hours (compare P1 and P2 in Fig. 3E with P1-2 in Fig. 3F). As expression of *Pax1* correlates with high levels of cell proliferation in the pharyngeal endoderm (Müller et al., 1996), our data suggest that 1-2BAFH and fusion of the corresponding aortic arches may result from the growth failure of the first pharyngeal pouch.

No alterations of the BAs, branchial clefts and pharyngeal pouches were observed upon treatment of wild-type embryos (n=15 in each group) with 10^{-7} M RARα-selective agonist (BMS753) or RARγ-selective agonist (BMS961) (compare B1, B2 and black arrowhead in Fig. 4B,E,H). However, both BMS753 and BMS961 retinoids were active, as they induced ectopic expression of an RA-inducible *Rare-hsp68-lacZ* reporter transgene (Rossant et al., 1991; Ang et al., 1996) in the frontonasal mass and in the tail, respectively (F and red arrow in Fig. 4).

BMS453 and TTNPB at concentrations that consistently induced 1-2BAFH in wild-type embryos (10^{-7} M and 5×10^{-9} M, respectively, see above), did not yield any abnormality in *Rarb*-null embryos (n=10 in each group; Fig. 5B,D; compare with Fig. 5A,C; data not shown). Altogether, these data indicated that the teratogenic effects of retinoids on BAs were specifically mediated by RARβ, provided that BMS453 actually acted as a bona fide retinoid in the embryo. To check this latter point, we used transgenic embryos carrying a *lacZ* gene controlled by the RA-inducible *Rarb2* promoter sequence (*Rarb2-lacZ* gene), which is active in some, but not all, embryonic tissues expressing the endogenous *Rarb* gene (Mendelsohn et al., 1991; Mendelsohn et al., 1994). In vehicle-treated control embryos (n=11), expression of the *Rarb2-lacZ* reporter was detected in the neural tube from the caudal neuropore up to the seventh rhombomere (R7, Fig. 6A). In embryos treated with 10^{-7} M BMS453 (n=9), this expression domain was shifted anteriorly, reaching the boundary between the third and the fourth rhombomeres (R3/R4 boundary; R3-4; Fig. 6B) (Mendelsohn et al., 1994). Adjunction to the culture medium of the panRAR-selective

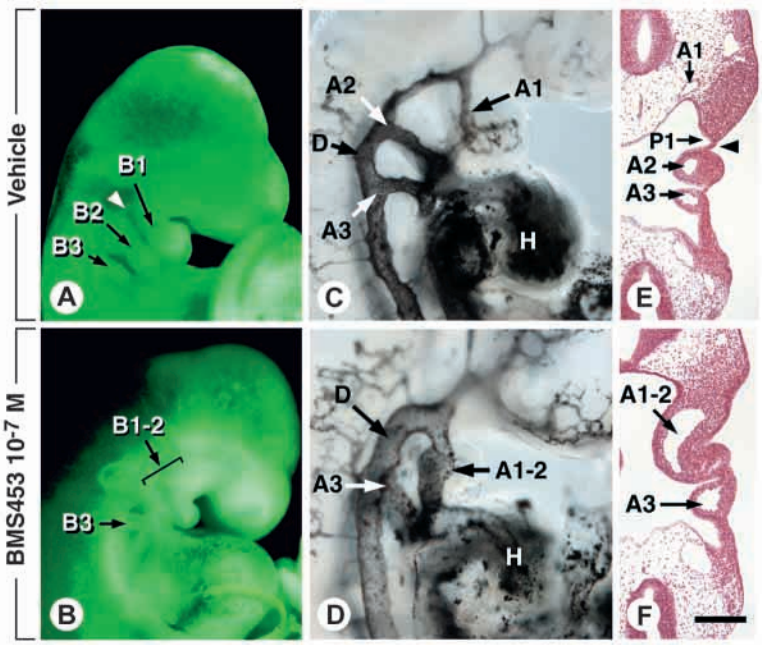

Fig. 2. Effects on branchial arch development of the RARβ agonist BMS453. External (A-D) and histological (E,F) aspects of the pharyngeal region of wild-type embryos after 48 hours in culture without retinoid (vehicle; A,C,E) or in the presence of the RARβ-selective agonist (B,D,F). Embryos in C and D were injected with ink. Frontal histological sections (E,F) were obtained from comparable levels of the embryos. A1-A3, aortic arches 1 to 3, respectively; A1-2 fused first and second aortic arches; B1-B3, branchial arches 1 to 3, respectively; B1-2, fused first and second branchial arches; D, dorsal aorta; H, heart; P1, first pharyngeal pouch. White arrowhead (A) indicates the first branchial cleft. Scale bar in F: 200 μm in E,F.

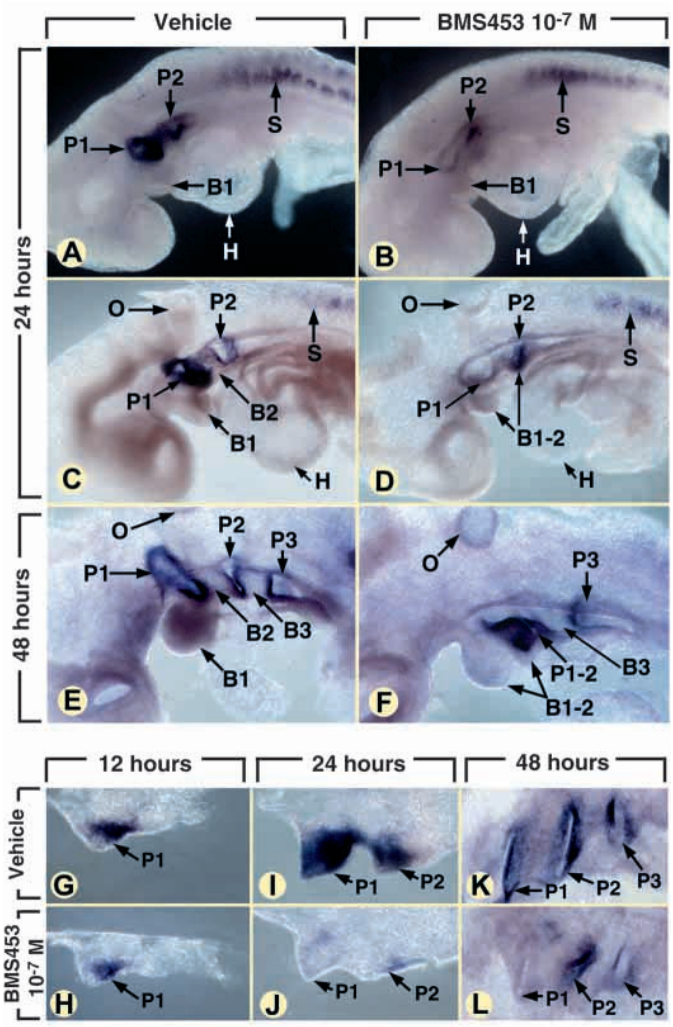

Fig. 3. Effects on branchial pouch development of the RARβ agonist BMS453. Embryos were hybridized with a *Pax1* antisense probe after 12, 24 and 48 hours in culture. (A,B) external views. (C-F) Embryos cut sagittally into halves; the medial side of the right half is displayed. (G-L) Ventral views of flat mounts from isolated pharyngeal endoderm. B1-B3, branchial arches 1 to 3, respectively; B1-2, fused first and second branchial arches; H, heart; O, otocyst; P1-P3, pharyngeal pouches 1 to 3, respectively; P1-2, fused first and second pharyngeal pouches; S, somites. A-F and G-L are displayed at the same magnification.

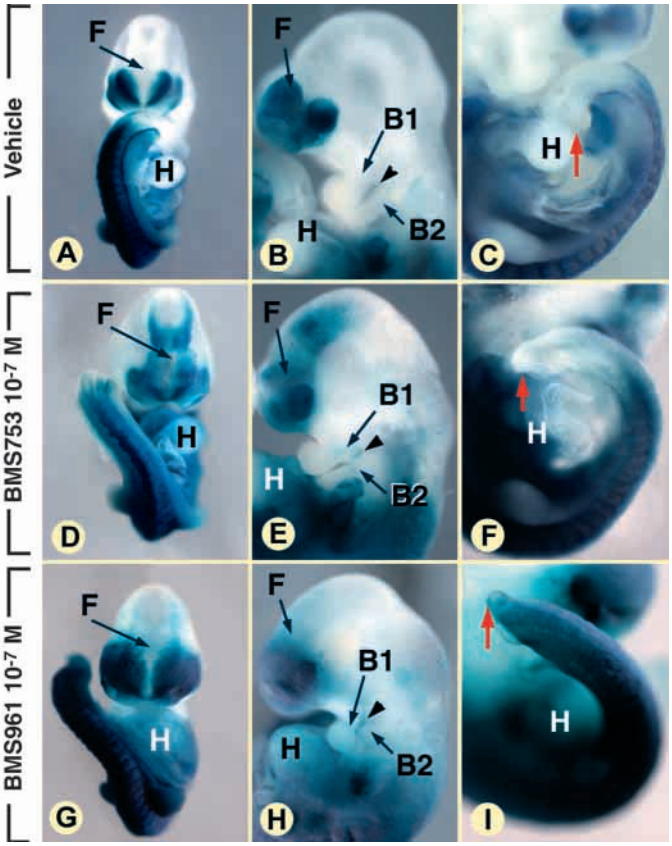

Fig. 4. The RARα-selective (BMS753) and the RARγ-selective (BMS961) agonists, induce ectopic expression of the *Rare-hsp68-lacZ* transgene in the frontonasal mass and tail, respectively, without altering branchial arch development. Ventral views (A,D,G) of wild-type embryos, and details of their pharyngeal (B,E,H) and caudal (C,F,I) regions, after 48 hours in culture either without retinoid (vehicle) or in the presence of isotype-selective RAR agonists. B1 and B2, branchial arches 1 and 2, respectively; F, frontonasal process; H, heart. Arrowheads indicate the first branchial cleft and red arrows point to the tips of the tails.

antagonist BMS493 at 10^{-6} M prevented not only the BMS453-induced ectopic expression of the *Rarb2-lacZ* transgene in the R4 to R6 territory (*n*=6; Fig. 6C), but also the generation of the 1-2BAFH (compare B1-2 in Fig. 6B with B1 and B2 in Fig. 6C). As expected, BMS493 also caused a general decrease of *Rarb2-lacZ* expression (compare Fig. 6A with Fig. 6C) (Wendling et al., 2000). These data indicate that the BMS453-induced 1-2BAFH is causally related to the activation of an RA signalling pathway.

Induction by BMS453 of ectopic RA signalling and RARβ expression in rhombencephalic neurectoderm and pharyngeal endoderm

To analyze tissue responsiveness to BMS453, we used embryos harbouring the *Rare-hsp68-lacZ* reporter transgene, whose expression pattern closely matches the distribution of endogenous RA, and which can be induced by exogenous RA in all tissues from E7.5 to E10.5 (Rossant et al., 1991; Ang et al., 1996). In control embryos (*n*=24), a robust expression of *Rare-hsp68-lacZ* was observed in the neurectoderm up to the R5/R6 boundary, whereas a weak β-galactosidase activity was detected in R5, but not in more anterior rhombomeres (Fig. 7A). *Rare-hsp68-lacZ* expression in the pharyngeal endoderm was restricted to its caudal part (pE, Fig. 7C,E) lining the presumptive third and fourth BAs. The anterior part of the pharyngeal endoderm (aE, Fig. 7C,E), lining the first and second BAs (B1 and B2, Fig. 7E) and forming the first pharyngeal pouch (P1, Fig. 7E), did not express the transgene. In embryos treated with 10^{-7} M BMS453 (*n*=16), *Rare-hsp68-lacZ* expression was strongly increased in R5, and became detectable in R4 (Fig. 7B). Concomitantly, a robust *lacZ* expression was observed throughout the whole pharyngeal endoderm (aE and pE, Fig. 7D,F), notably in the region lining the fused branchial arches (B1-2, Fig. 7F). However, *lacZ* expression was not induced in the ectoderm covering the first

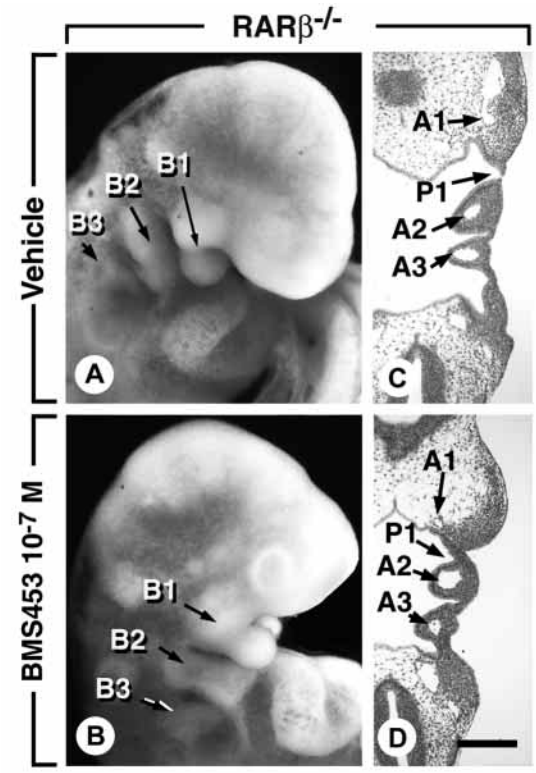

Fig. 5. *Rarb*-null embryos are resistant to BMS453-induced teratogenic effects. External views (A,B) and frontal histological sections (C,D) of the branchial arches of *Rarb*-null embryos after 48 hours in culture either without retinoid (vehicle) or in the presence of BMS453. A1-A3 and B1-B3, aortic arches and branchial arches 1 to 3, respectively; P1, first pharyngeal pouch. Scale bar in D: 200 μm for C,D.

two BAs (EC, Fig. 7E,F), in the mesenchyme (M1, M2 and M1-2, Fig. 7E,F), or in the neural crest cells (NCCs), which, after 24 hours in culture, are still migrating into the nascent second BA (brackets, Fig. 7G,H). Note that treatment of *Rarb*-null embryos carrying the *RARE-hsp68-lacZ* transgene with 10^{-7} M BMS453 did not affect the pattern of *lacZ* expression in the pharyngeal endoderm (aE, compare Fig. 7H with Fig. G,I), or in the hindbrain (data not shown). These results indicate that treatment with BMS453 specifically triggers ectopic expression of an RA-inducible transgene in R4 and in the endoderm of the first and second BAs.

Expression of *Hoxa1* and *Hoxb1* in the endoderm of the primitive gut and in the hindbrain neurectoderm is under RA control (Marshall et al., 1996; Wendling et al., 2000). In vehicle-treated embryos, these genes were expressed in the posterior part of the pharyngeal endoderm (pE, Fig. 8A,B) but not in its anterior part (aE, Fig. 8A,B). In addition, *Hoxb1* was expressed in R4 (R4, Fig. 8B). Treatment with BMS453 induced strong, ectopic expression of both genes in the anterior part of the pharyngeal endoderm (aE, Fig. 8C,D) in addition to ectopic expression of *Hoxb1* in the dorsal region of R3 (R3, Fig. 8D). This ectopic transcriptional activation of RA-target genes indicates that BMS453 triggers a RARβ-mediated RA-signalling pathway at a level along the anteroposterior body axis corresponding to the first and second BAs.

Between E8.0 and E9.5, which corresponds to the developmental period covered by our culture experiments, RARβ transcripts are undetectable in the tissues of the first two BAs and in the neural crest cells from which the BA mesenchyme originates (Ruberte et al., 1991; Mollard et al., 2000). However, the RARβ promoter, which contains a functional RARE (de Thé et al., 1990), can be activated in various embryonic tissues upon exposure to pharmacological doses of retinoids (Harnish et al., 1990; Mendelsohn et al., 1991; Osumi-Yamashita et al., 1992). We therefore assumed that the generation of 1-2BAFH could be accounted for by ectopic activation of a RARβ-mediated signalling pathway. In control embryos (n=8), RARβ transcripts were detected in the caudal rhombencephalon up to R6 (Fig. 9A), as well as in the posterior part of the pharyngeal endoderm (pE, Fig. 9C). By contrast, RARβ transcripts were absent from R5 and more anterior rhombomeres (Fig. 9A), as well as from the anterior part of the pharyngeal endoderm (aE, Fig. 9C). Upon treatment with 10^{-7} M BMS453 (n=8), the RARβ expression domain was shifted anteriorly, now encompassing R4 and R5 (Fig. 9B), as well as the anterior part of the pharyngeal endoderm (aE, Fig. 9D). Thus, altogether these findings demonstrate that the RARβ-specific agonist BMS453 can induce ectopic expression of RARβ and of other RA-target genes along the neuroectodermal and endodermal territories located at a level along the anteroposterior body axis where BMS453-induced developmental defects are occurring.

The RARβ agonist BMS453 synergizes with the panRXR agonist BMS649 and antagonizes other RAR isotypes

Embryos cultured in the presence of BMS453 at 10^{-8} M (n=32) were undistinguishable from controls (compare A1-A3, P1 and arrowhead in Fig. 10A and Fig. 2E) and, as already mentioned above, exposure to the panRXR agonist BMS649 at 10^{-6} M (n=18) did not yield embryonic defects (compare A1-A3, P1 and arrowhead in Fig. 10B and Fig. 2E). By contrast, all embryos treated with a combination of 10^{-8} M BMS453 and 10^{-6} M BMS649 (n=22) displayed 1-2BAFH, as

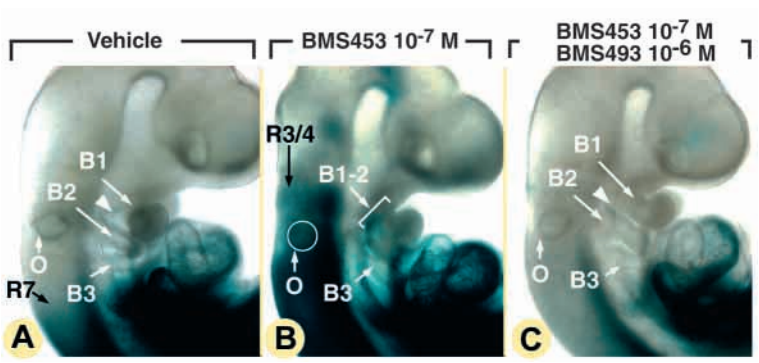

Fig. 6. BMS453-induced branchial arch defects and ectopic activation of the RARβ2 promoter are prevented by the panRAR-selective antagonist BMS493. Embryos carrying the *Rarb2-lacZ* transgene in a wild-type genetic background were cultured for 48 hours. B1-B3, branchial arches 1 to 3, respectively; B1-2, fused first and second branchial arches; O, otocyst; R7, rhombomere 7; R3/R4, boundary between rhombomeres 3 and 4. Arrowheads indicate the first branchial cleft.

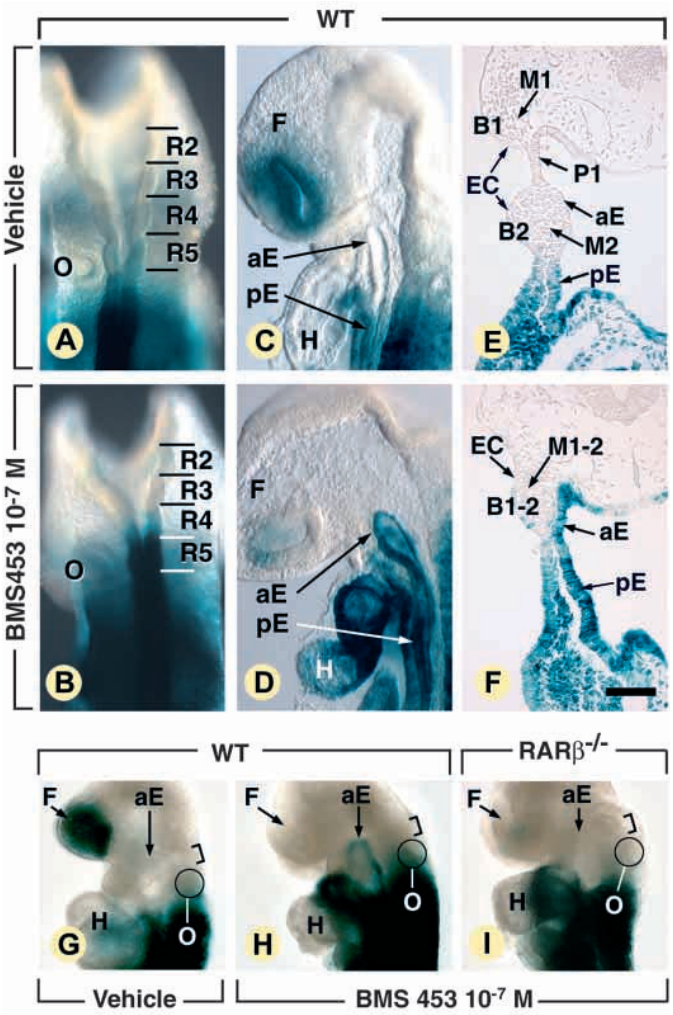

Fig. 7. Expression of *lacZ* in vehicle-treated and BMS453-treated embryos carrying the *Rare-hsp68-lacZ* transgene in a wild-type (WT; A,H) and in an *Rarb*-null (*Rarb*$^{-/-}$; I) genetic background, after 24 hours (A-D,G-I) or 30 hours (E,F) in culture. (A,B) Dorsal views of intact embryos. (C,D) Embryos cut sagittally into halves; the medial side of the right half is displayed. (E,F) Frontal histological sections through the pharynx. (G-I) Lateral views of intact embryos. Brackets (G-I) indicate the putative localization of migrating NCCs destined to the second BA. B1-B2 and M1-M2, branchial arches 1 and 2, and their mesenchyme, respectively; B1-2 and M1-2, fused first and second branchial arches and their mesenchyme, respectively; aE and pE, anterior and posterior regions of the pharyngeal endoderm, respectively; EC, ectoderm; F, frontonasal process; H, heart; O, otocyst; P1, first pharyngeal pouch; R2-R5, rhombomeres 2 to 5, respectively. Scale bar in F: 100 μm for E,F.

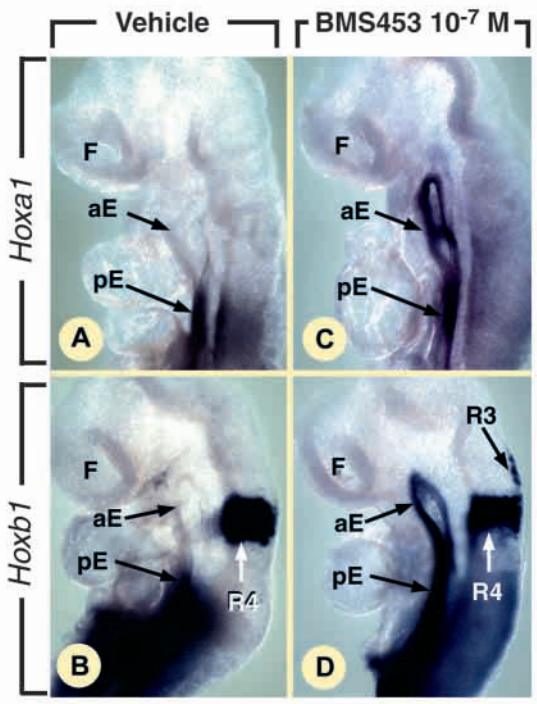

Fig. 8. Expression of *Hoxa1* and *Hoxb1* in vehicle-treated and BMS453-treated wild-type embryos after 24 hours in culture. All embryos were cut sagittally into halves and the medial side of the right half is displayed. aE and pE, anterior and posterior parts of the pharyngeal endoderm, respectively; F, frontonasal process; R3 and R4, rhombomeres 3 and 4, respectively.

well as a fusion of the corresponding aortic arches (A1-2 in Fig. 10C; data not shown). Therefore, non-teratogenic concentrations of BMS649 and BMS453 can synergistically induce malformations generated by a teratogenic concentration of BMS453 on its own (compare A1-2 in Fig. 10C and Fig. 2F).

Treatment with BMS453 not only caused ectopic activation of RARβ signalling in R4 (Fig. 6B, Fig. 7B, Fig. 9B) and in the anterior part of the pharyngeal endoderm (aE, Fig. 7D,F,H, Fig. 8C,D, Fig. 9D, Fig. 11B), but also consistently induced a decrease of *Rare-hsp68-lacZ* expression in the frontonasal mass (F) and tail (T) of the embryo (compare Fig. 7G with Fig. 7H, and Fig. 11A,D,G with Fig. 11B,E,H). On histological sections, this decreased expression was seen: (1) in the forebrain (FO), optic vesicle (OV) and the mesenchyme (M) surrounding them (compare Fig. 11D and E); and (2) in the tail tissues (T) except for the mesonephric duct (D, compare Fig. 11A and B; data not shown). However, it is noteworthy that all these tissues can respond to an activation of RAR signalling as both RA (Rossant et al., 1991) and TTNPB increased their *Rare-hsp68-lacZ* transgene expression (compare Fig. 11A,D,G with Fig. 11C,F,I). Furthermore, the BMS453-induced decrease of *Rare-hsp68-lacZ* expression was clearly not mediated by RARβ as it was also observed in *Rarb*-null embryos treated with BMS453 (compare F in Fig. 7H,I; data not shown). Altogether, these results indicate that BMS453, aside from its RARβ agonistic properties, can antagonize signalling pathways mediated by the other RARs in vivo, namely RARα and/or RARγ (see Discussion below).

DISCUSSION

Treatment of embryos with retinoids (retinol or RA) induces multiple malformations, the nature and spectrum of which depend both on the dose and on the developmental stage at the time of exposure (reviewed by Soprano and Soprano, 1995; Collins and Mao, 1999). Evidence that teratogenic effects of retinoids are receptor mediated has been inferred: (1) from

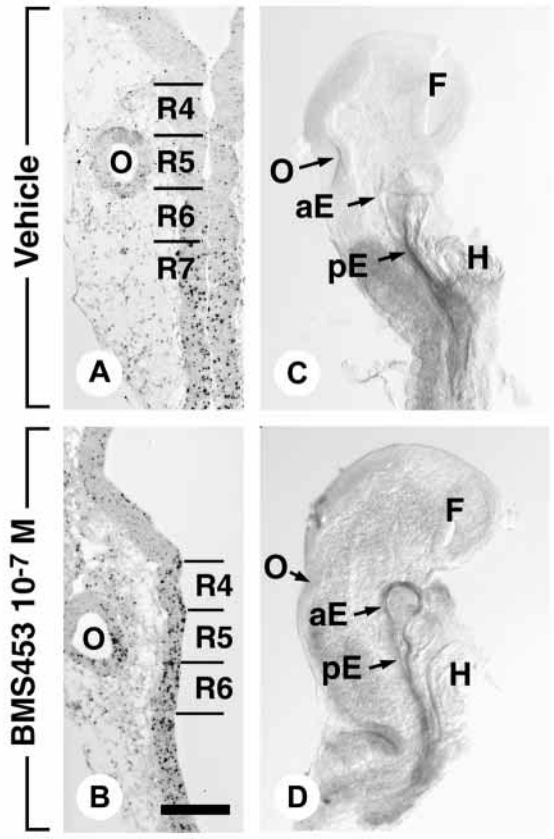

Fig. 9. Distribution of mRARβ transcripts in vehicle-treated and BMS453-treated wild-type embryos after 24 hours in culture. (A,B) Frontal histological sections through the rhombencephalon. (C,D) Embryos hybridized in toto with the mRARβ probe cut sagittally into halves; the left medial side is displayed. aE and pE, anterior and posterior parts of the pharyngeal endoderm, respectively; F, frontonasal process; H, heart; O, otocyst; R4-R7, rhombomeres 4 to 7, respectively. Scale bar in B: 150 μm for A,B.

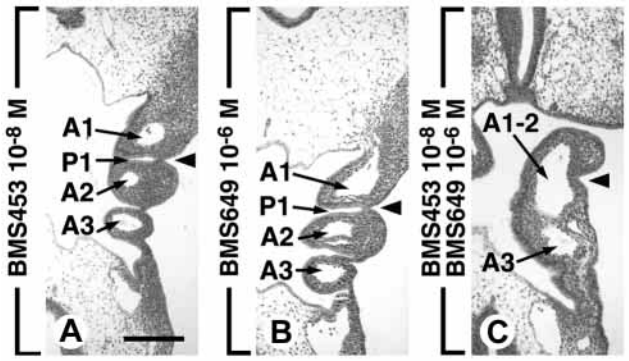

Fig. 10. Synergistic effects of the RARβ agonist BMS453 and of the panRXR-selective agonist BMS649. Frontal histological sections are from comparable levels of wild-type embryos after 48 hours in culture. A1-A3, aortic arches 1 to 3, respectively; A1-A2 fused first and second aortic arches; P1, first pharyngeal pouch. Arrowhead indicates the first branchial cleft. Scale bar in A: 200 μm for A-C.

comparison of the teratogenic potency of synthetic retinoids and their ability to activate their cognate receptors; and (2) from RA treatment of animals overexpressing or carrying null mutations of retinoid receptors. First, it has been shown that synthetic retinoids that are unable to activate RARs are non-teratogenic (Kochhar et al., 1996), whereas RAR isotype-selective retinoids produce a distinct spectrum of defects (Elmazar et al., 1996; Arafa et al., 2000). Second, overexpression of RARγ in *Xenopus* embryos potentiates the RA-induced loss of cranial structures, whereas expression of a dominant-negative form of RARs results in a resistance to RA-induced malformations (Old et al., 1996; Blumberg et al., 1997; van der Wees et al., 1998). Along the same lines, both *Rarg*- and *Rxra*-null mouse embryos are resistant to some RA-induced malformations (Lohnes et al., 1993; Iulianella and Lohnes, 1997; Sucov et al., 1995; Nugent et al., 1999). RARα/RXR heterodimers could participate in RA-induced teratogenesis, as the frequency and/or severity of some morphological defects is increased by co-administration of an RARα-selective and an RXR-selective agonist (Lu et al., 1997; Elmazar et al., 1997; Elmazar et al., 2001). In the present study, we show that a characteristic RA-induced defect of craniofacial development is mediated by RARβ/RXR heterodimers, in which the activity of RXR is subordinated to that of RARβ, and also show that the primary target tissue of RA-induced teratogenicity is most probably the branchial arch (BA) endoderm. In addition, we provide the first in vivo evidence that an RARβ-selective agonist can also antagonize RARα and RARγ isotypes.

RARβ/RXR heterodimers mediate teratogenic effects of RA in the branchial region of the head

We show that the fusion and hypoplasia of the first two BAs (1-2BAFH), which results from RA administration at a developmental stage equivalent to E8.0 in the mouse (Webster et al., 1986; Goulding and Pratt, 1986; Lee et al., 1995; Wei et al., 1999), can also be generated by treatment with the panRAR-selective agonist TTNPB. Furthermore, TTNPB-induced 1-2BAFH is not observed in *Rarb*-null embryos, and can be mimicked in wild-type embryos by treatment with the RARβ-selective agonist BMS453 but not with an RARα-selective or RARγ-selective agonist. These findings strongly support the conclusion that abnormal activation of RARβ signalling is both necessary and sufficient to induce the typical early teratogenic effect induced by RA administration at E8.0. External and middle ears derive from the first branchial cleft ectoderm, the first pharyngeal pouch endoderm and the mesenchyme located at the interface between the first and second BAs, whereas mandibular bone differentiates from the first BA mesenchyme (Larsen, 1993). Thus, ear and mandible deficiencies that, in human newborns, are hallmarks of RAE (Lammer et al., 1985; Coberly et al., 1996), could be accounted for by an abnormal activation of RARβ signalling during the fourth week of pregnancy (equivalent to E8.0 in the mouse).

In cultured cells, RAR- and RXR-selective agonists act synergistically to promote cell proliferation, differentiation or apoptosis through transactivation of RA-responsive genes. Moreover, the ligand-bound RXR is transcriptionally active only if its RAR partner is also ligand-bound (the so-called RXR subordination) (Lotan et al., 1995; Roy et al., 1995; Horn et al., 1996; Taneja et al., 1996; Botling et al., 1997; Chiba et al., 1997; Minucci et al., 1997; Altucci and Gronemeyer, 2001).

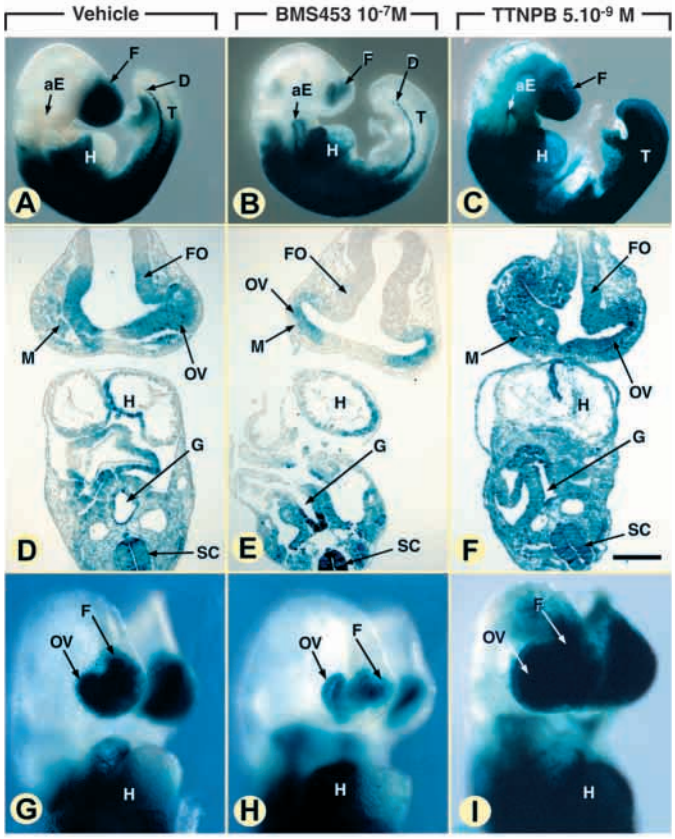

Fig. 11. Treatment with the RARβ agonist BMS453 decreases RA signalling in the frontonasal process and tail. External views (A-C,G-I) and frontal histological sections (D-F) of embryos carrying the *Rare-hsp68-lacZ* transgene in a wild-type genetic background. Transgenic embryos were cultured for 24 hours (G-I) or 30 hours (A-F) in the presence of vehicle (A,D,G), BMS453 (B,E,H) and the panRAR-selective agonist TTNPB (C,F,I). aE, anterior part of the pharyngeal endoderm; F, frontonasal process; FO, forebrain; D, mesonephric duct; G, foregut; H, heart; M, mesenchyme of the frontonasal process; OV, optic vesicle; SC, spinal cord; T, tail. Scale bar in F: 400 μm for D-F.

Along the same lines, RXR-selective ligands, which are inactive on their own, synergize in vivo with RARα- and RARγ-selective ligands to generate congenital malformations (Kochhar et al., 1996; Lu et al., 1997; Elmazar et al., 1996; Elmazar et al., 1997; Elmazar et al., 2001). In our experiments, the panRXR agonist BMS649 (SR11237) had no effect on its own, indicating that neither RXR homodimers, nor other heterodimers in which the RXR partner is transcriptionally active on its own, mediate the teratogenic effects of retinoids on the branchial region. However, the RXR-selective ligand can reveal the dysmorphogenetic effects of the RARβ agonist at a concentration that, on its own, is not teratogenic. Thus, retinoid-induced teratogenesis of BAs is mediated through ectopic activation of RARβ/RXR heterodimers, in which the ligand-dependent activity of RXR is subordinated to that of its ligand-bound RARβ partner.

The pharyngeal endoderm is a primary target tissue of RA-induced teratogenesis

Because of the multiplicity and complexity of malformations induced upon exposure to RA, target tissues of RA-induced teratogenicity are difficult to identify. Several observations have suggested that the cranial neural crest represents such a target tissue. First, almost all the structures that are malformed in human RAE are derived from neural crest cells (NCCs) migrating through BAs (Larsen, 1993). Second, analyses of animal models indicate that excess RA can alter NCC survival and migration. For example, NCC apoptosis that occurs as a result of RA administration to mouse embryos is a probable cause of the craniofacial defects observed in newborns (Sulik et al., 1988; Alles and Sulik, 1992). Along the same lines, aberrant NCC migration after RA treatment has been correlated with hypoplasia of the first BA in rat embryos (Lee et al., 1995).

Thus, RAE apparently meets the criteria for a neurocristopathy, i.e. ‘a condition arising from aberrations of the early migration, growth and differentiation of NCCs’ (Bolande, 1997). However, retinoid-induced fusion of the first and second BA occurs without alterations of NCC migration or apoptosis (Lee et al., 1995). Moreover, we show that NCCs contributing to the first two BAs do not express a retinoid-responsive transgene upon treatment with BMS453. Therefore, under our experimental conditions, NCCs are not the primary targets of retinoid-induced teratogenicity. Interestingly, recent evidence also indicates that NCCs may not respond directly to RA under physiological conditions (Dupé et al., 1999; Wendling et al., 2000; Iulianella and Lohnes, 2002; Jiang et al., 2002).

By contrast, treatment with BMS453 disrupts the development of the first two pharyngeal pouches and triggers ectopic RARβ-dependent RA signalling in the endoderm lining the first two BAs, manifested by the rostral shift of the expression domains of RA-responsive genes (i.e. the *Rare-hsp68-lacZ* reporter, the *Rarb* gene, the *Hoxa1* and *Hoxb1* genes). Hox gene expression probably plays an important role in anteroposterior regionalization of the pharyngeal endoderm (Mulder et al., 1998; Manley and Capecchi, 1998; Wendling et al., 2000), which can be set up even in the absence of NCCs (Veitch et al., 1999; Gavalas et al., 2001; Escriva et al., 2002; Graham and Smith, 2001). Conversely, pharyngeal endoderm plays a seminal role in the formation of BAs, through imparting patterning information to NCCs (Couly et al., 2002), as well as to ectodermal cells (Begbie et al., 1999). In our BMS453-treated embryos, impaired development of the first pharyngeal pouch probably occurs as a direct consequence of improper regionalization of the pharyngeal endoderm. As BA segmentation cannot proceed without pharyngeal pouches (Piotrowski and Nusslein-Volhard, 2000), the growth failure of the first pouch might account for the fusion of the first two BAs in embryos exposed to retinoids at a developmental stage equivalent to mouse E8.0. Interestingly, treating E9.0 mouse embryos with RA leads to disruption of the third pharyngeal pouch and branchial cleft, along with a fusion of the third and fourth BAs, which is accompanied by a marked increase of *Hoxa3* expression in the pharyngeal endoderm (Mulder et al., 1998). These data and our present findings support the view that craniofacial, thymic and cardiovascular defects observed in RAE, as well as in genetically determined neurocristopathies (Jerome and Papaioannou, 2001), can all result from an abnormal function of the pharyngeal endoderm.

BMS453 acts in vivo as an RARβ-selective agonist displaying RARα- and RARγ-selective antagonistic properties

Retinoids (notably RA) are widely used in cancer chemoprevention, as well as for treating oncological and dermatological diseases (Lotan, 1996; Nason-Burchenal and Dmitrovsky, 1999). However, undesirable side-effects, including toxicity and teratogenicity, are observed upon treatment with RA, most probably because of the panRAR and panRXR agonistic activity of RA (Orfanos et al., 1987). The use of synthetic agonists that selectively interact with given receptor isotypes is expected to reduce such side-effects. In situ hybridization analyses (Ruberte et al., 1991) have established that the three RARs display distinct expression profiles at E8.5 (i.e. just a few hours earlier than the developmental stage illustrated in Figs 7 and 11). During this time period, both neurectoderm and mesenchyme of the head region strongly express RARα, whereas the tail tissues strongly express RARγ. RARβ transcripts are present in trunk tissues and in mesonephric duct, but are not detectable in the forebrain and tail regions. The overlap between (1) the expression domains of RARα, β and γ during normal embryonic development, and (2) the distribution of responsive cells in RARα-selective agonist BMS753-, RARβ-selective agonist BMS453- and RARγ-selective agonist BMS961-treated embryos indicates that RA signals are transduced preferentially by RARα in tissues of the embryonic head, by RARβ in tissues of the embryonic neck, and by RARγ in tissues of the embryonic tail. Interestingly, BMS453-induced ectopic activation of retinoid signalling in the hindbrain and BA endoderm is accompanied by a decrease of signalling in the forebrain and tail regions (Fig. 11). A similar decrease is observed in the forebrain and tail regions upon treatment with a panRAR antagonist (Wendling et al., 2000), as well as in embryos carrying a null mutation of the RA-generating enzyme RALDH2 (Niederreither et al., 1999). Therefore, this decrease results from a block in RA signalling and not from a toxic effect of BMS453, which definitely acts, in vivo, as a bona fide RARβ-selective agonist displaying an RARα and RARγ antagonistic activity. This is in keeping with data obtained in vitro in stably transfected cells (Chen et al., 1995).

Interestingly, anteriorization of RARβ expression triggered by BMS453 has to be mediated by RARβ itself and not by other RARs as: (1) BMS453 antagonizes RARα and RARγ signalling in the embryo; and (2) treatment of RARβ-null embryos carrying the *Rare-hsp68-lacZ* transgene with BMS453 does not affect the pattern of *lacZ* expression in the pharyngeal endoderm (Fig. 7). Thus, even though the levels of RARβ transcripts are below the threshold for detection in the anterior pharyngeal endoderm (Fig. 9), RARβ has to be present in this region where it can induce its own promoter if activated by a ligand. This observation is in keeping with data obtained from cultured cells (Roy et al., 1995; Taneja et al., 1996; Chiba et al., 1997). Many pre-malignant and malignant cells exhibit a reduced RARβ expression (Sun et al., 2000), whereas forced-recovered expression of RARβ in breast cancer cells restores RA-induced growth arrest and apoptosis (Seewaldt et al., 1995). As the RARβ-selective agonist BMS453 does not activate RARα and RARγ, it could be less toxic than RA, and therefore of therapeutic value in the treatment of cancers characterized by reduced RARβ expression.

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