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Pharmacological Activity of Retinoic Acid Receptor Alpha-Selective Antagonists in Vitro and in Vivo

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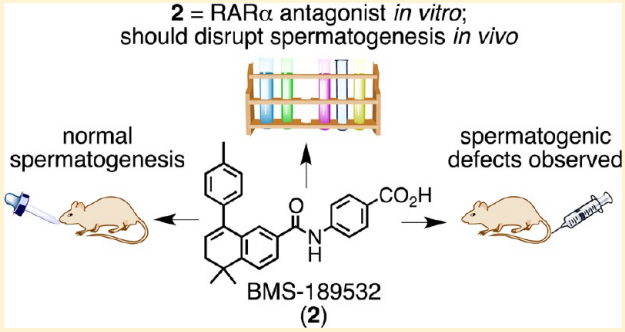
Supporting Information

ABSTRACT: Oral administration of a retinoic acid receptor (RAR) pan-antagonist reversibly inhibits spermatogenesis. Given the importance of RARα in regulating spermatogenesis, we identified two RARα-selective antagonists by transactivation and transactivation competition assays and asked whether they effectively inhibit spermatogenesis. Although these two antagonists were potent in vitro, they displayed poor in vivo activity in mice when administered orally. Testicular weights were normal, and morphological analysis revealed normal spermatid alignment and sperm release. In vitro drug property analyses were performed with one of these antagonists and compared with the pan-antagonist. We showed that the discrepancies may be explained by several factors, including high plasma protein binding, faster hepatic metabolism relative to the pan-antagonist, and only moderate permeability. The conclusion of poor oral bioavailability was supported by more pronounced defects in mice when the antagonist was administered intravenously versus intraperitoneally. These results are crucial for designing new RARα-selective antagonists for pharmaceutical application.



KEYWORDS: *Retinoic acid receptor alpha, antagonist, spermatogenesis, drug property profiling*

Classic studies on vitamin-A-deficiency (VAD) resulting in mammalian male sterility revealed the important role of vitamin A and its physiologically active metabolite all-*trans*-retinoic acid (ATRA) in normal spermatogenesis.[1−3] The predominant function of retinoids is due to their binding as ligands to retinoic acid receptors (RARs) or retinoid X receptors (RXRs).[4] Using targeted mutagenesis in mouse models, deletion of the mouse *Rara* gene led to male sterility and defects in spermatogenesis that resembled VAD testes,[5−7] including a failure of sperm alignment and release into the tubular lumen. Targeted expression of *Rara* exclusively in haploid spermatids of RARα-deficient mice was shown to partially rescue spermatogenesis and restore fertility,[8] suggesting a crucial role of RARα-mediated retinoid signaling during spermiogenesis.

Various synthetic retinoids have been produced to identify cellular responses to retinoid signaling.[9,10] Bristol−Myers Squibb (BMS) and other companies developed a series of low-molecular-weight arotinoid compounds that function as RAR antagonists by blocking ATRA binding and activation of transcription of RAR target genes (Figure 1).[11−13] Using

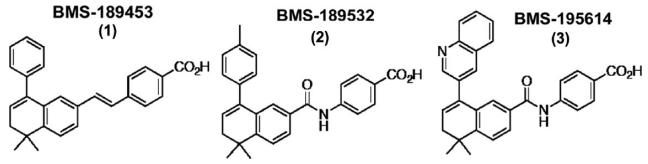


Figure 1. Structures of RAR antagonists.

systematically modified dosing regimens, we demonstrated that low levels of RAR pan-antagonist BMS-189453 (**1**) inhibited spermatogenesis in mice without any observable side-effects.[13] The cellular processes involved in spermiogenesis and completion of spermiation were extremely sensitive to changes in retinoid signaling induced by this pharmacological intervention, resembling abnormalities seen in VAD and RARα-deficient testes. Importantly, the induced sterility was reversible, with a full recovery of spermatogenesis,[13] suggesting

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that RAR-antagonists may represent new lead molecules in developing nonsteroidal male contraceptives.

We therefore wished to explore the use of RARα-selective antagonists as a pharmacological approach to inhibit spermatogenesis in mice. As our concomitant genetic studies have demonstrated that RARα is essential for spermatogenesis,[5−8] we speculated that such drugs would inhibit spermatogenesis, possibly at lower doses than the pan-antagonist.

Two antagonists, BMS-189532 (**2**) and BMS-195614 (**3**) (Figure 1), were characterized as RARα-selective by transactivation and transactivation competition assays. These two compounds were considered to be retinoid antagonists as they inhibited ATRA-induced RARE-CAT (chloramphenicol acetyltransferase) reporter expression by concomitantly transfected RARs, but did not, by themselves, induce reporter expression (Supporting Information, Figure S1A-C for **3**). The transactivation properties of **2** have been previously published[14] and were found to be very similar to **3**, consistent with observations by others.[15] Further, **2** and **3** demonstrated selectivity for RARα by competition assays (Figure 2A,B, respectively). Interestingly, **2** appears to exhibit greater RARα-selective antagonistic activity than **3** (Figure 2A versus B).

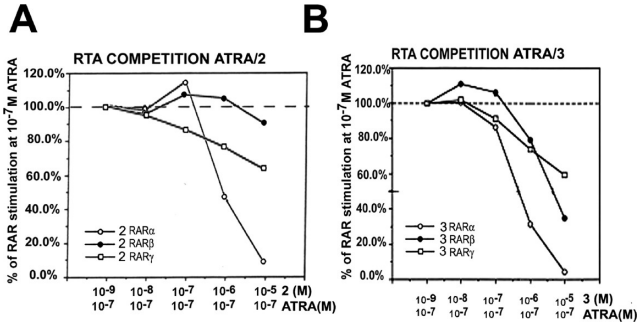

Figure 2. Characterization of RAR antagonists. Transactivation competition assay for assessment of antagonist activity. CAT reporter activity was measured in the presence of 10^{-7} M ATRA and increasing concentrations of each retinoid. Both **2** (A) and **3** (B) selectively compete ATRA-induced CAT expression for RARα (open circles) with minimal activity observed for RARβ (closed circles) and RARγ (squares) and are thus RARα-selective antagonists. The internal cotransfection control is a plasmid containing a constant beta-galactosidase concentration. Vehicle controls were the dilution medium without retinoid. ATRA was used as a positive control.

Given the similar testicular abnormalities induced by the oral administration of pan-antagonist **1** to those observed in *Rara*$^{-/-}$ mice[13] and the essential role of RARα during spermatogenesis shown by our genetic studies,[5−8] we speculated that these RARα-selective antagonists would also have marked effects on spermatogenesis. No toxicological or other in vivo studies examining testicular histology in animal models for these drugs have been published. Oral doses of 2.0 and 10 mg/kg for 7 days were chosen to minimize possible bioavailability differences.[10,16] Surprisingly, detailed morphological analysis of testes obtained one-month after the 7-day dose revealed no effect on spermatogenesis (Figure 3). Specifically, alignment of step 16 spermatids at the lumen in stage VIII tubules and sperm release at stage IX into the lumen appeared normal in testes from mice treated with both compounds in both the 2 mg (data not shown) and even with the 10 mg group (**3**, Figure 3B; **2**, Figure 3E), compared with control (Figure 3A,D). In addition, neither vacuolar-like

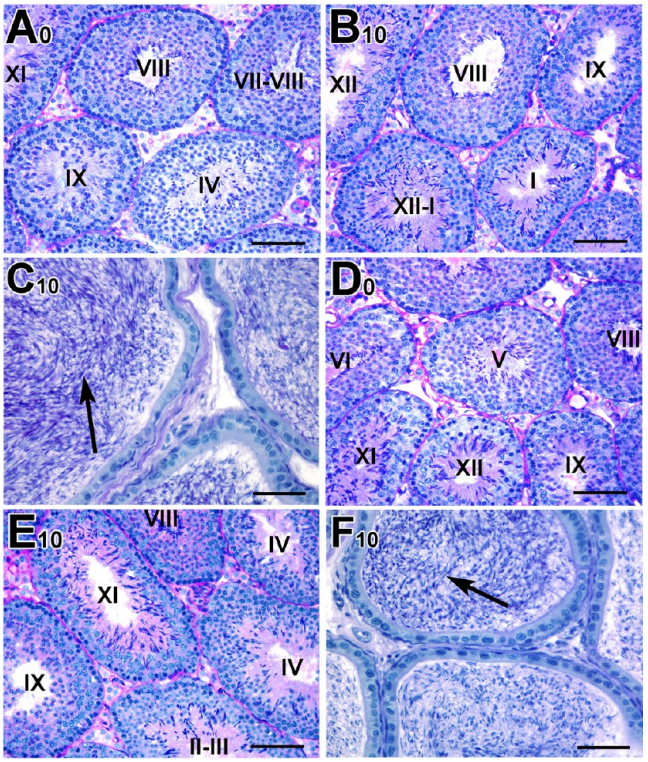

Figure 3. Lack of inhibition of spermatogenesis by oral doses of RARα-selective antagonists at 1 month post-treatment with **2** and **3**. Histological sections of mice treated with vehicle alone (A,D) and 10 mg/kg for 7 days of **3** (B,C) and **2** (E,F). A,B,D,E, testes; C,F, epididymides. Roman numerals indicate the stage of tubules. Scale bar: 50 μm. Arrows point to the spermatozoa in the epididymides.

spaces nor asynchronization of spermatogenic cell association were observed (Figure 3B,E). Further, the morphology of caudal epididymides appeared normal (arrow, **2**, Figure 3C; arrow, **3**, Figure 3F), and epididymal sperm exhibited normal motility (data not shown).

No changes in body weight of the drug-treated mice compared with controls were observed throughout the study at any of the dosing regimens (data not shown). Concomitant with the lack of effect on spermatogenesis, no statistically significant changes were observed in testicular weights of either the 2 mg or the 10 mg group, compared with control (Figure 4A). As expected, none of the dosing regimens changed testosterone levels (Figure 4B), similar to our previous observations.[13] The observed variation in testosterone levels is typical in mice,[17] compared with relatively consistent levels in both rats and humans and did not differ between drug-treated and control samples.

Thus, although these two antagonists were potent in vitro, both compounds were clearly ineffective in vivo by oral administration. To begin to explore why these two antagonists exhibited such limited efficacy in vivo, we synthesized a sample of **2**, which was spectroscopically identical to **2** from BMS.[14] This tolyl-containing compound was selected over the quinolinyl-containing **3** for its ease of synthesis[14] and because it had greater RARα-selective antagonistic activity (Figure 2). The procedure for synthesizing **2** was modified from a published procedure[14] (Supporting Information). The chemical synthesis of **1** was described previously.[13]

To assess properties that might have resulted in the poor in vivo efficacy of these compounds, Cerep, Inc. (Redmond, WA)

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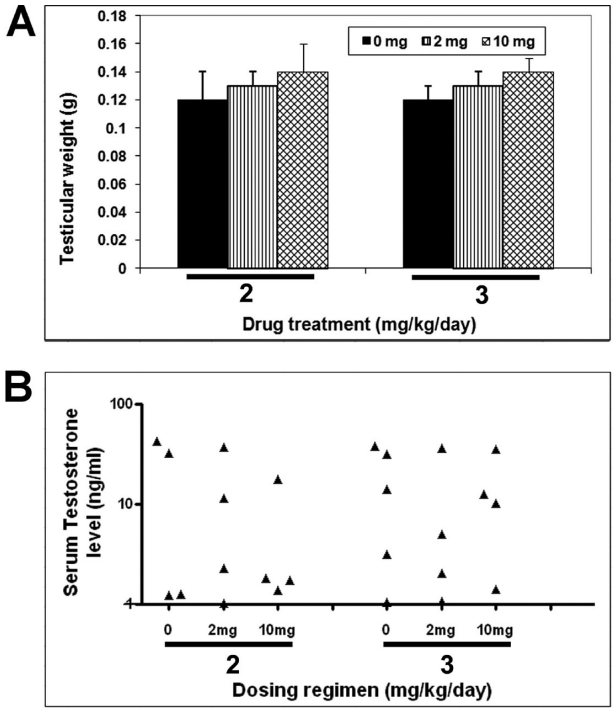

Figure 4. Gonad weight and serum testosterone. A: The testicular weight of **2**- and **3**-treated mice at different dose treatments. The bars represent the mean ± SD of five mice for each regimen. No significant differences within age group-matched mice with different treatments compared with control as assessed by paired Student's *t*-test. B: Males were treated with **2** and **3** at two different regimens, and sera were obtained for determination of testosterone level. Data points from individual mice are presented.

performed the following drug property analyses with RARα-selective antagonist **2** and RAR pan-antagonist **1**: HPLC-mass spectrometry; three solubility property assays (aqueous solubility, partition coefficient, and plasma protein binding); an in vitro absorption and efflux assay (A−B and B−A permeabilities); and an in vitro liver microsome metabolic stability assay. An additional intestinal microsome stability assay was performed by XenoGesis, Inc. (Nottingham, UK). HPLC−MS confirmed that both compounds had not degraded during storage or transit, with purity at the time of analyses being 99%. Two of the five in vitro drug property assays would have predicted (seemingly erroneously) that **2** should have had higher bioavailability than **1** (Table 1). The ability to be absorbed via the human intestinal mucosa, as determined in a Caco-2 cell model (pH gradient of 6.5/7.4) was significantly lower for **1** than for **2** (Student's *t*-test, $p < 0.001$). The A−B permeability for **1** was $<0.7 \pm 0.04 \times 10^{-6}$ and $8.3 \pm 0.4 \times 10^{-6}$ cm/s for **2**. Thus, **2** has only moderate permeability and the 20% recovery shows nonspecific binding, degradation, or retention in the cells. Analysis of B−A permeability displayed little efflux of **2** ($0.7 \pm 0.1 \times 10^{-6}$ cm/s). Retinoid **1** was also considerably less soluble than **2** (3.1 ± 0.2 versus 136 ± 0.9 μM, respectively, in PBS buffer at pH 7.4, $p < 0.001$). The aqueous solubility of **2** was moderately high (136 ± 0.9 μM; a range of <1−200 μM). The partition coefficients (log *D*) for both compounds are within the preferred range for orally bioavailable drugs (<5). The weighted average log *D* value for **1** was 3.25 and 3.84 for **2** at pH 7.4 using *n*-octanol and PBS buffer. The moderate permeability plus moderately high solubility of **2** should have been high enough to be bioavailable at the 2 mg dosing, but not high enough to ensure its full bioavailability at the 10 mg dosing.

Table 1. In Vitro Drug Property Analyses of 1 and 2

assay (conditions)	1	2
aqueous solubility[a]	3.1 ± 0.2 μM	136 ± 0.9 μM
partition coefficient[b]	3.25	3.84
plasma protein binding[c]	95.3 ± 3.1% bound; 93.3 ± 24.0% recovery	99.2 ± 0.9% bound; 99.8 ± 5.5% recovery
A-B permeability[d]	$<0.7 \pm 0.04 \times 10^{-6}$ cm/s; 14% recovery	$8.3 \pm 0.4 \times 10^{-6}$ cm/s; 20% recovery
B-A permeability[d]	nc[g]	$0.7 \pm 0.1 \times 10^{-6}$ cm/s; 46% recovery
metabolic stability (liver)[e]	79 ± 13.6% parent remaining	49 ± 1.3% parent remaining
metabolic stability (intestine)[f]	nc[g]	86 ± 7.0% parent remaining

[a]PBS, pH 7.4. [b]log *D*, *n*-octanol/PBS, pH 7.4, 1.0×10^{-4} M, weighted average of 3 trials. [c]CD-1 mouse, test at 1.0×10^{-5} M. [d]Caco-2, pH 6.5/7.4, 1.0×10^{-5} M. [e]liver microsomes, CD-1 mouse, 1.0×10^{-6} M, 60 min. [f]Intestinal microsomes, CD-1 mouse, 1.0×10^{-6} M, 60 min. [g]nc = not calculated.

While the drug property assays showed that **1** has poor solubility and permeability compared with **2** (Table 1), the combination of two properties of **2** (plasma protein binding and metabolic stability) can perhaps explain in part the in vitro and in vivo discrepancies although the differences were not significant at the 5% level in and of themselves. The plasma protein binding assay showed that 99.2 ± 0.9% of **2** was protein bound with 99.8 ± 5.5% recovery using CD-1 mouse plasma. Nonetheless, **1** had a more favorable plasma protein binding value (95.3 ± 3.1% of **1** was protein bound with 93.3 ± 24.0% recovery using CD-1 mouse plasma, Table 1). Only 0.8% of **2** was not plasma protein bound and that very small portion of free compound was presumably metabolized at a much faster rate than **1**. The metabolic stability assay using CD-1 mouse liver microsomes showed that **2** is less hepatically stable, compared with **1** (49 ± 1.3% versus 79 ± 13.6% of the parent compound remained after 60 min, respectively). When **2** was subjected to an intestinal microsome stability assay, there was little metabolism observed (86 ± 7.0% remained after 60 min). The presence of high levels of drug metabolizing enzymes in the liver following oral dosing could have resulted in extensive metabolism of compounds that would lead to their low oral bioavailability.[18] Therefore, **2** is more rapidly metabolized, yielding a shorter half-life, compared with **1**.

Accordingly, the effect on spermatogenesis of **2** administered intravenously (IV) and intraperitoneally (IP) was examined. Acute disruptive effects of **2** on spermatid alignment and sperm release into testicular lumen at the end of drug treatment were found (Figure 5C,D) after IV administration for 6 ($n = 2$) and 7 days ($n = 2$). These abnormalities were similar to those seen in testes of mice treated orally with **1**. No effect on spermatogenesis was found in all males 4-weeks post-treatment (data not shown) indicating the effect was reversible. Similar defects were only found in some tubules of males administered intraperitoneally ($n = 4$; Figure 5E,F). Quantitative analysis of these tubules revealed that more pronounced defects were found in mice after **2** was administered intravenously versus intraperitoneally observed one day post-treatment (Supporting Information, Figure S3 and Table S1), which supports the possibility that poor oral bioavailability of **2** is responsible in part for the lack of effect on spermatogenesis.

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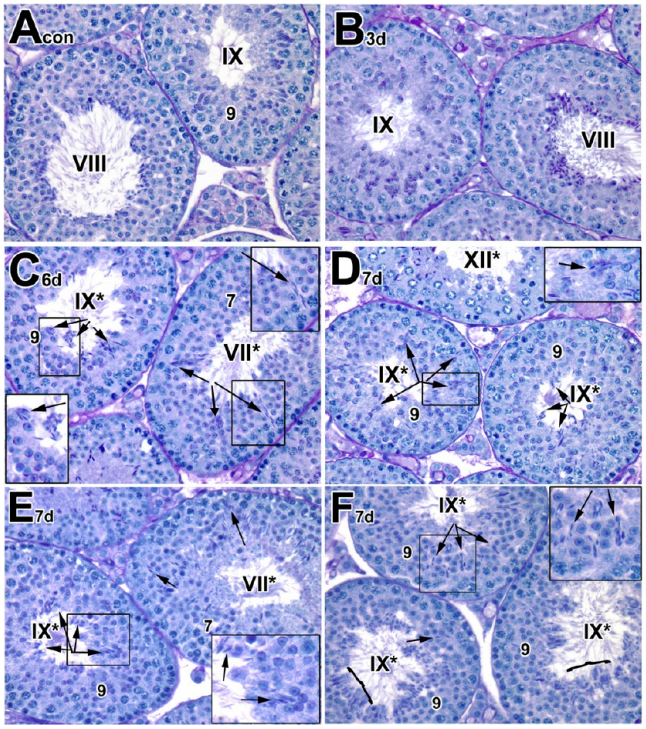

Figure 5. Acute disruptive effect of **2** on spermatid alignment and release in testicular tubules immediately after IV and IP administration. Histological sections of testes of mice treated with vehicle alone (A) or with 10 mg/kg of **2** administered intravenously for 3 days (B), 6 days (C), and 7 days (D) or intraperitoneally for 7 days (E,F). Testes were processed one day post-treatment. Magnification: 40×. Arabic numerals, the step of spermatid differentiation; Roman numerals, the stage of tubules. Although abnormal cell associations complicate staging, an attempt was made to stage drug-treated tubules using the acrosomal system, and tubules are labeled with a Roman numeral followed by an asterisk. Arrows or curved bracket point to the retained spermatids.

Finally, the testicular bioavailability of **2**, assessed by serum and tissue pharmacokinetic analyses, further supported our hypothesis. Mice were dosed with **2** (oral or IV), and the concentration of **2** in plasma and testes was measured (Supporting Information, Figure S2A,B). Overall, the IV group exhibited higher testicular bioavailability because the areas under the curve (AUCs) showed a higher percentage of **2** crossing the blood-testis barrier compared with the oral-dosing group (AUC = 2941 versus 2268).

Male contraception is an essential component of worldwide reproductive health but is poorly developed.[19,20] A need exists for an effective, reversible nonhormonal male contraceptive with minimal side effects. Our previous study clearly demonstrated that pan-antagonist **1** can impair spermatogenesis reversibly and without any detectable side effects.[13] In the present study, clearly, but unexpectedly, we showed that although two RARα-selective compounds were potent in vitro, they were ineffective in disrupting spermatogenesis in mice at oral doses similar to **1**.[13] While we were initially surprised to see both compounds without an effect at the doses tested, subsequently obtained archived data from BMS indicated that almost an order of magnitude higher dose (75 mg/kg) was required to see effects on spermatogenesis in Wistar rats (C. Zusi, personal communication), consistent with our observations in mice. Such high-dose regimens would clearly be undesirable for future drug development.

Given the importance of retinoid compounds in inhibiting spermatogenesis, we were interested in understanding the biochemical and physiological basis for the discrepancy between the in vitro and in vivo efficacy. Properties including solubility, permeability, metabolic stability, and transporter effects are important for the success of drug candidates because they affect oral bioavailability, metabolism, clearance, toxicity, and in vitro pharmacology.[21,22] Traditionally, drug discovery programs have been driven largely by potency, regardless of their in vivo properties, which can result in developing costly nondrug-like molecules with high risk and low success rate.[22] Our current analysis illustrates an example of a class of antagonists that were potent in vitro but were ineffective when administered orally. As such, our studies provide structure–property relationships that are essential to guide structural modification to improve critical properties when designing new RARα-selective antagonists for pharmaceutical application.

In summary, we have demonstrated that although RARα-selective antagonists BMS-189532 (**2**) and BMS-195614 (**3**) were active in vitro, they displayed poor in vivo activity in mice when administered orally. We have further determined that for **2** this discrepancy is due to poor oral bioavailability, which may be the result of several factors, including high plasma protein binding and faster hepatic metabolism relative to **1** and additionally only moderate permeability.

EXPERIMENTAL PROCEDURES

See the Supporting Information for detailed methodologies. Antagonists **2** and **3** obtained from BMS were used in a transactivation assay as described previously.[13,23,24] Antagonistic activity of the compounds was determined by a competition assay. Using the same constructs described previously,[13] HeLa cells were incubated with a submaximal dose of ATRA and with increasing concentrations of the test compound, and CAT expression was measured after 24 h.

The in vivo test was performed using CD1 mice (Charles River Laboratories). Compounds **2** and **3** were administered by oral gavage to males at daily doses of 2 and 10 mg/kg for 7 days as described previously.[13] Control received vehicle alone for 7 days. The testes were collected and weighed one month post-treatment for morphological assessment of the effect of the compound on spermatogenesis. Compound **2** was also administered by IV and IP to males daily at 10 mg/kg for 7 days to assess the effect on spermatogenesis.

ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures, materials and apparatuses, additional details for animal experiments, and the chemical synthesis of **2** data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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