Non-Steroidal Progesterone Receptor Specific Ligands

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Abstract: The nuclear receptor for progesterone is a target for contraception and for several therapeutic indications. Progestin agonists and antagonists in clinical use mimic the steroidal backbone of the cognate ligand, progesterone. Thus, they have significant cross-reactivity with other steroid receptors. Recently, non-steroidal progesterone receptor ligands have begun to appear in the literature. This review will describe the current status of research into these promising new chemical entities.

INTRODUCTION

The progesterone receptor, like other steroid receptors, plays a unique and crucial role in mammalian development and homeostasis. Progesterone is known to be required for mammary gland development, ovulation and the maintenance of pregnancy. It may have less clearly defined functions in bone, the cardiovascular system and the central nervous system. In terms of opportunities for pharmacological intervention, it is arguably the sex steroid receptor with the greatest unexploited potential. Currently, steroidal progestin agonists and antagonists are clinically approved for contraception, hormone replacement therapy and therapeutic abortion. But there is good preclinical and clinical evidence for the value of progestin antagonists in treating endometriosis, uterine leiomyomata (fibroids), dysfunctional uterine bleeding and breast cancer. There are several new compounds in late-phase clinical trials that are going to impact these therapeutic areas in the coming years. Some of these are non-steroidal compounds, which tend to be significantly more specific for their target receptor than steroids, and thus to potentially have a more favorable therapeutic ratio. Non-steroidal progesterone receptor ligands will likely emerge as major players in reproductive pharmacology in the foreseeable future.

This review will focus on recent developments in research on the biology of progesterone and its receptors, and it will describe novel progesterone receptor ligands that have emerged from chemistry and pharmacology laboratories in the past few years. For comprehensive previous reviews of this area, the reader is referred to several recent publications [1-7].

NEW INSIGHTS IN PROGESTERONE RECEPTOR BIOLOGY

Since the description of what came to be known as progestational activity in the early part of the last century [8], major milestones in progesterone research included the isolation of the active substance in the 1930s [9] and the cloning of the receptor in the 1980s [10]. Another milestone was reached in 1995 with the generation of a "knockout" mouse lacking functional progesterone receptor [11]. Studies of the consequences of this mutation on the physiology of these animals have provided significant new information. In addition, a clearer, if still far from complete understanding of the molecular mechanisms of gene regulation by the progesterone receptor has emerged in the past decade.

Physiology of PR Knockout Mice

The first progesterone receptor knockout mouse (PRKO) lacked both the A- and B-forms of the receptor (Fig. **1**) [11]. Both male and female mice carrying the PRKO null mutation developed to adulthood, but the females were far from normal, having major functional defects in all reproductive tissues (Table **I**). They were unable to ovulate, because otherwise normal follicles failed to rupture in response to luteinizing hormone. They did not respond sexually in the presence of a male, a result that suggested a neuroendocrine role of the hormone in sexual receptivity. Later work showed that gonadotropin regulation was disrupted in PRKO mice, and that, unlike wild type mice, mutant females did not exhibit a luteinizing hormone surge following exposure to male odor [12]. The uteri of the knockout animals were hyperplastic and prone to inflammation. Epithelial hyperplasia in the uterus is an expected consequence of unopposed estrogen action. Finally, PRKO mammary ductal epithelium did not proliferate in response to progesterone, nor did lobuloalveolar differentiation occur, although mammary glands developed normally during puberty [11, 13].

More recent work with male PRKO mice pointed to a role of the hormone in regulating male sexual behavior and aggression. Null mutant mice without sexual experience mounted less often than inexperienced wild type males, while experienced PRKO animals lost sexual activity following castration, unlike wild type animals. In addition, heterozygous mutants were less sexually responsive to androgens [14]. In another study [15], PR knockouts lost the infanticidal behavior typical of male mice, while administration of progesterone to wild type males exacerbated aggression towards infant mice. Conversely, antagonism of endogenous progesterone in wild type mice reduced aggression. Aggression directed towards other adults was unaffected by progesterone or its receptor.

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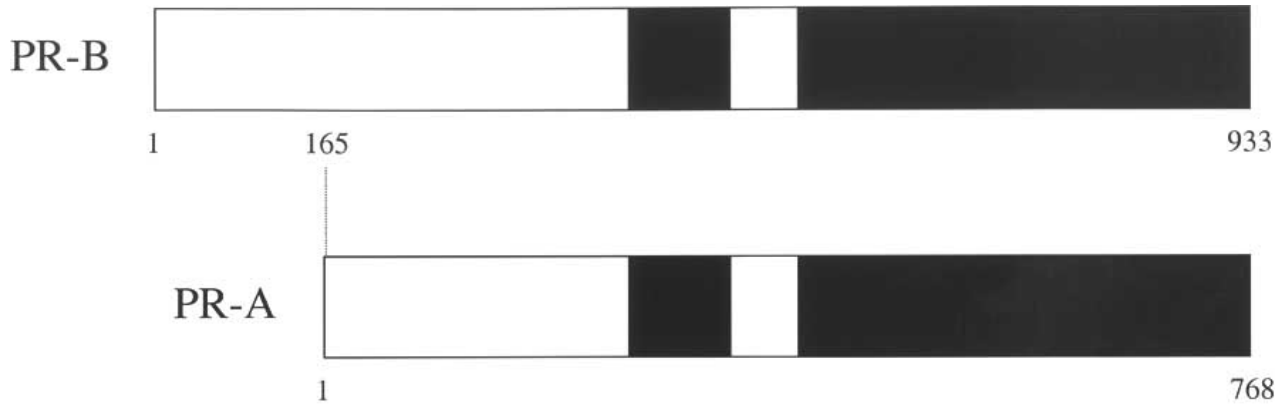


Fig. (1). Schematic representation of the two isoforms of the human progesterone receptor. Amino acid numbers are shown below each figure. DBD: DNA-binding domain. LBD: ligand binding domain.

Karas *et al.* [16] studied the effect of the PR null mutation on the response to vascular injury. Compared to wild type, ovariectomized knockout animals showed increased vascular medial hypertrophy and smooth muscle cell proliferation following injury to the carotid artery. Exogenous progesterone worsened the response to injury in wild type mice. When vascular smooth muscle cells were cultured *in vitro*, those from PRKO mice were hyperproliferative, while wild type cells were growth-inhibited by progesterone. Transient reintroduction of the progesterone receptor gene to PRKO cells restored progesterone-mediated inhibition, which could in turn be reversed by a progesterone antagonist. These results point to a possible role of progesterone in the response to vascular injury.

Physiological Roles of PR-A and PR-B

After they were defined [17,18], it was something of a mystery what functional differences existed between the full-length progesterone receptor isoform (PR-B) and its shorter counterpart (PR-A) (Fig. **1**). Molecular studies showed that the PR-A isoform had little transactivation activity by itself in transfected cells in culture, but was able to inhibit the activity of the B-isoform [19] and of other steroid hormone receptors, including the estrogen receptor [20,21]. The divergent activities of the two isoforms could be due to differential recruitment of coregulators – PR-A was better able to bind the corepressor SMRT and less able to bind coactivators than PR-B *in vitro* [22].

New results with mouse knockouts specific for the PR-A isoform (PRAKO) or the PR-B isoform (PRBKO) showed that the *in vivo* situation is, not unexpectedly, more complex. In the uterus, the epithelial hyperplasia observed in PRKO mice was maintained in PRAKO mice treated with estrogen (Table **I**) [23]. Thus, PR-A is required to oppose the proliferative effect of estrogen in the uterus. Unexpectedly, when PRAKO uteri treated with estrogen and progesterone were examined, proliferation was further increased. This indicated that PR-B is stimulatory in these cells, and that PR-A counteracts both the estrogen receptor and PR-B in wild type uterine epithelial cells. These results nicely complemented the *in vitro* description of PR-A negative regulatory activity detailed above.

However, PR-A is not merely a dominant negative regulator, even in uterine cells. This is because it is still required for maintaining uterine receptivity in PRAKO mice, as measured by testing for decidualization of stromal cells following estrogen and progesterone treatment [23]. Moreover, positive expression of a subset of genes associated with implantation (amphiregulin and calcitonin) was lost upon mutation of PR-A. Likewise, ovulation is disrupted in PRAKO mice, albeit not as drastically as in the PRKO knockout animal. Like PRKO, PRAKO mutants are not fertile.

Table I. Summary of Phenotypes in Progesterone Receptor Null Mutant Mice

Phenotype	PRKO	PRAKO	PRBKO
Fertility	No	No	Yes
Ovulation	None	Reduced	Normal
Uterine receptivity	None	None	NR
Uterine proliferation	Epithelial hyperplasia	Epithelial hyperplasia	NR
Mammary gland	No response to P	Normal	Impaired proliferation
Sexual receptivity (female)	None	NR	NR
Sexual activity (male)	Reduced	NR	NR
Infanticide (male)	Reduced	NR	NR

PRKO: PR-A and PR-B knockout. PRAKO: PR-A knockout. PRBKO: PR-B knockout. P: progesterone. NR: not reported

In contrast to its roles in the uterus and ovary, PR-A does not appear to be required for progesterone-dependent proliferation and differentiation in the mouse mammary gland [23]. Presumably, the defective phenotype in PRKO is due to loss of the PR-B isoform. Results with PRBKO mice confirm this, although there is an indication that the phenotype in this tissue is not identical between the two mutants; specifically, progesterone-dependent proliferation of ductal cells is reduced, while alveolar differentiation is not. The latter may require both isoforms [24]. Finally, ovulation is normal in PRBKO mice, confirming the necessary and sufficient role of PR-A in this process [24]. PRBKO mice are fertile, implying the same for uterine implantation [24,25].

The Progesterone Receptor and Breast Cancer

Progestin antagonists remain of interest as potential new hormonal agents for the treatment of breast cancer, despite slower than expected development over the past two decades [26]. Disappointing clinical results with mifepristone [27] and liver toxicity with onapristone [26] were serious setbacks. But new progestin antagonists are under development, and combination treatment regimens, such as with estrogen antagonists, may be more effective.

Expression of PR in breast tumors is considered a sign of estrogen receptor α expression, and hence of a more favorable prognosis [28]. Histochemical evaluation of breast tissue showed that the expression of PR-A tended to be higher than that of PR-B in malignant lesions compared to normal breast [29]. It is difficult to know the functional significance of this, given the dominant negative action of PR-A over PR-B discussed above. PR-A appears to be dispensable for normal breast proliferation in mice [24]; this may not hold true in humans. Alternatively, alteration of the ratio of the two isoforms may disrupt epithelial proliferation in ways we have yet to understand. Adult mice over-expressing the PR-A isoform exhibit increased ductal branching [30], while those over-expressing PR-B have retarded lateral branching without an effect on alveolar growth [31]. In the mouse PR null mutants, we do not know the consequences of ablation of either isoform on mammary tumorigenesis. However, *in vitro*, mammary glands from PRKO mice are resistant to the carcinogenic effects of 7,12-dimethylbenza[a]nthracene on normal epithelia [32].

Research has begun into the roles of steroid receptor coactivators and corepressors in mammary tumorigenesis. Coactivators are factors that interact with nuclear receptors to enhance their transcriptional activity, and corepressors interact with nuclear receptors to reduce their transcriptional activity [33]. Three major classes of coactivators have been identified – SRC-1, SRC-2 and SRC-3. SRC-3 (also known as AIB1, for Amplified In Breast cancer 1) was originally identified as an amplified gene in 10% of primary breast tumors [34]. It was over-expressed in 64% of the tumors analyzed. In mice, it is more specifically expressed than the other two coactivators, with strong expression in the mammary gland [35]. Mice lacking the SRC-3 gene had growth defects and a number of reproductive abnormalities, including retarded mammary gland development [36]. SRC-1 null mutants also had mammary defects, exhibiting retarded ductal morphogenesis [37]. Other coactivators are over-expressed in breast tumors, including E6-AP [38,39] and SRA [40]. While preliminary, these data suggest that coactivators may be involved in breast cancer development at some level. There is less evidence for an involvement of corepressors, but it is worth pointing out that BRCA1, one of the breast cancer susceptibility genes, can act as a corepressor for the estrogen receptor α, the androgen receptor and PR [41].

CHEMISTRY

Since there are a number of review articles published in this area for the period before 2000 [4-7], this section will focus on the development of non-steroidal progestin receptor modulators over the past three years. During this period, there were no additional reports on the three earliest series of non-steroidal progestins, tetrahydropyridazines (**1**) (Fig. **2**),

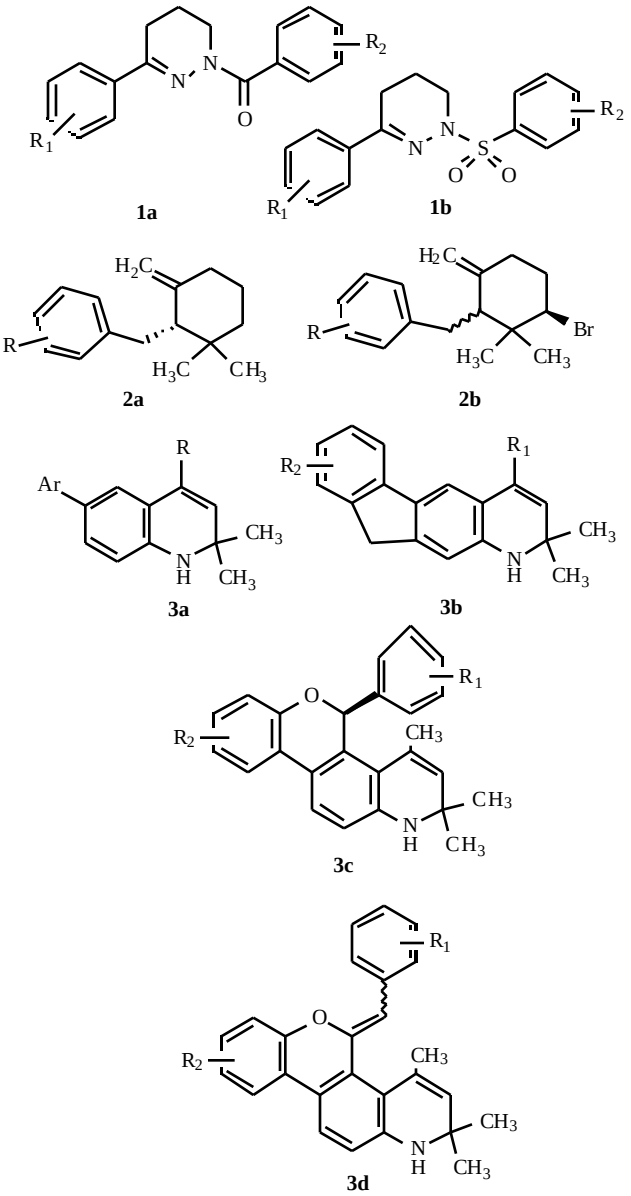


Fig. (2). Previously identified non-steroidal progestin receptor ligands. Tetrahydropyridazines (**1**), cyclocymopol derivatives (**2**) and dihydroquinoline derivatives (**3**).

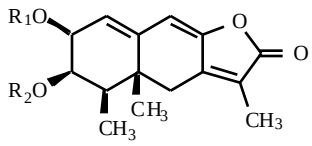
cyclocymopol derivatives (**2**) and dihydroquinoline derivatives (**3**). It is worth noting that androgen receptor modulators and glucocorticoid receptor modulators were also identified from analogs derived from the dihydroquinolines. Divergent structure-activity relationships (SARs) have been developed for PR, the androgen receptor and the glucocorticoid receptor from structures related to these templates.

The eremmophilane-type sesquiterpenes (**4**) (Fig. **3**) have received more attention. The first members of this class of progesterone receptor modulators were isolated as bacterial metabolites from *Penicillium obtalum* seven years ago [42,43]. Subsequent synthetic studies enabled more SAR development on this class of natural products [44-46]. Reports on SAR information about these analogs, as well as their functional activity on the progesterone receptor and the *in vivo* efficacy of some of the analogs has emerged [46-49]. As shown in (Fig. **3**), methyl and ethyl at the 3-position (R_3) decrease the binding affinity to PR, while methyl is tolerated at 4-position. Eliminating the methyl at the 5-position dramatically decreased the binding affinity, whereas replacing the methyl with methoxy maintained potency. Similarly to the 3-position, a methyl is not tolerated at the 9-position. Interestingly, an α-methyl at the 7-position displayed at least equal potency to the β-hydroxy analog [46]. Biological studies demonstrated that for the substitution on the oxygen atom at the 6-position, both large acyl groups such as propionyl and furoyl, and a carbamate such as cyclopropyl carbamate are either tolerated or increase potency [47,48]. For example, compound **CP8481** had a relative binding affinity (progesterone = 100) of 150 against human PR and compound **CP8401** had an RBA of 15, while PF1092A had an RBA of 15 in the same assay. Like most of the analogs in this series, both compounds had weak androgen receptor binding affinity and virtually no affinity to the glucocorticoid receptor or the estrogen receptor [47].

Tetrahydrobenzindolone analogs such as **CP8661, CP8668** and **CP8863** (Fig. **4**) have received some attention [49,50]. **CP8668** showed potent binding to human PR with an RBA of 92 (progesterone = 100, mifepristone = 72) and low affinity to rat androgen receptor (RBA = 0.25, testosterone = 100), and no affinity to human glucocorticoid receptor and human estrogen receptor. **CP8661** had a lower affinity to human PR (RBA = 4.1, mifepristone = 100).

In *in vitro* functional assays, both tetrahydrobenzfuranones and the tetrahydrobenzindolones were identified as progesterone receptor antagonists or partial agonists. PF1092A and PF1092B are partial agonists as determined in human mammary carcinoma T47D cells; whereas CP8401 is an antagonist, albeit a less potent one than mifepristone (IC_{50} = 40 nM *versus* 1.4 nM). CP8661 and CP8668 also showed antagonistic activity in T47D cell-based functional assays. CP8661 had an IC_{50} of 110 nM, compared with 1.1 nM for mifepristone. CP8668 is a partial agonist in the cell assays.

A few derivatives of these natural products have been tested *in vivo.* In the rabbit endometrial transformation test, CP8661 showed anti-progestational effects at 10 mg/kg, when given subcutaneously (s.c.) [48]. Interestingly, CP8668 showed good progestational activity in the rabbit endometrial transformation assay with a McPhail index of 3.6 (at a dose of 5 mg/kg, s.c.), 2.3 (at a dose of 5 mg/kg, when given orally [p.o.]) and 3.2 (at a dose of 10 mg/kg, p.o.) [49]. In mice, CP8816 inhibited estradiol-induced mitotic activity in the uterine luminal epithelium, suggesting a progesterone-like antiproliferative effect on the



	R_1	R_2
PF1092A	H	Ac
PF1092B	Ac	H
PF1092C	H	H

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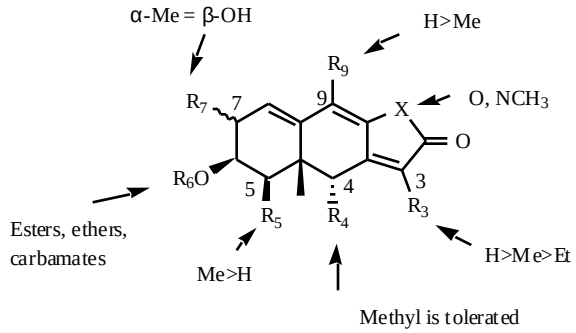
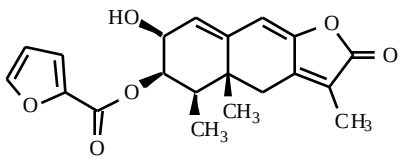
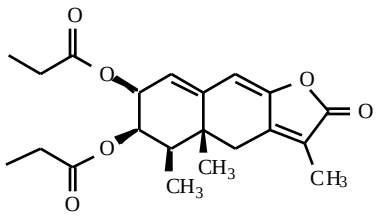


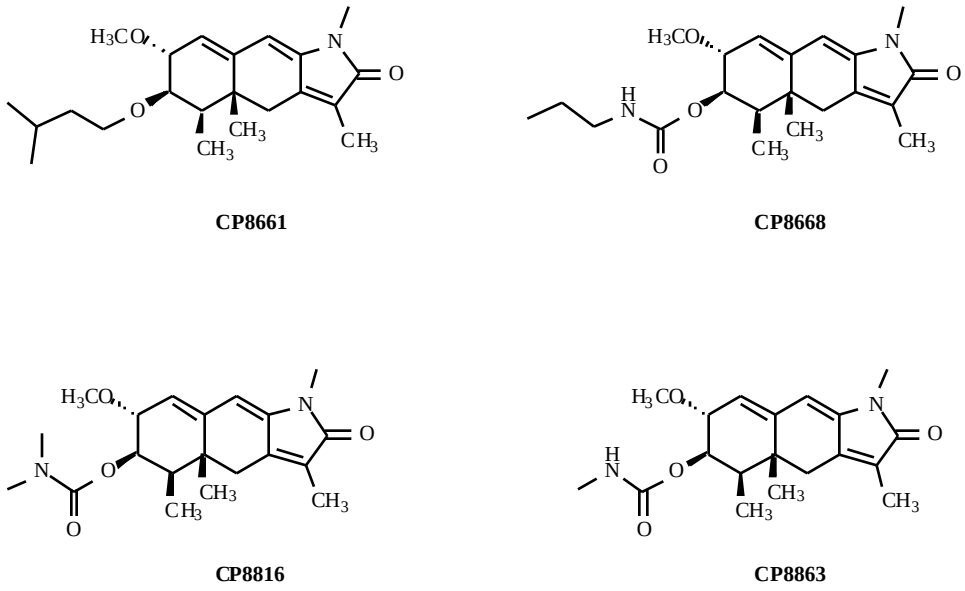


CP8481



CP8401

Fig. (3). Preliminary PR structure-activity relationship for sesquiterpene compounds.



CP8661 **CP8668**

CP8816 **CP8863**

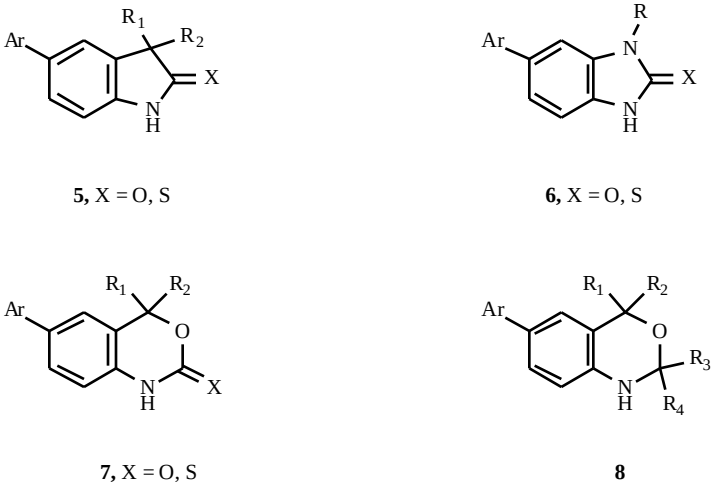
Fig. (4). Tetrahydrobenzindolone analogs.

epithelial component. However, it did not have an effect on stromal cells [50]. It was suggested that CP8816 and CP8863 could be therapeutic inhibitors of the development of adenomyosis.

More structures derived from the dihydroquinolines (**3**) (Fig. **2**) have emerged in the literature [51-56]. These new structures can be roughly summarized in the following four series (Fig. **5**): indolones (**5**), benzimidazolones (**6**), benzoxazinones (**7**) and benzoxazines (**8**). Interestingly, for structures **5** and **7**, when X is oxygen, the compounds are usually progesterone receptor antagonists while their thio derivatives (X = S) exhibit potent agonist activity. In general, the agonists in these series appeared to be more potent than the antagonists, based on functional assays in T47D cells. The agonists in these series are probably the most potent non-steroidal PR agonists reported so far.

A typical analog in the indolone series, **5a** (Fig. **6a**), had an IC_{50} of 14 nM in blocking progesterone-induced alkaline phosphatase activity in T47D cells. This compound also showed antagonistic activity in rat decidualization assays at 3 mg/kg p.o. However, the efficacy of **5a** only reached 70%, which is less efficacious than mifepristone. A close thio-derivative, **5b**, exhibited very potent PR agonist activity, with an EC_{50} of 0.36 nM in the T47D cell-based functional assay. This compound also had potent agonist activity in a rat decidualization assay (ED_{50} = 0.1 mg/kg) and rat complement component C3 assay (ED_{50} = 0.025 mg/kg) by the oral route. Many other indole-2-thiones in this series exhibited good *in vitro* and *in vivo* efficacy as PR agonists [51,52].

The benzimidazolones (**6**) (Fig. **6b**) were also identified as PR antagonists as part of an effort to improve the potency of the dihydroquinoline analogs **3a**. In PR binding assays, the benzimidazol-2-thiones (X = S) seemed to possess higher affinity than benzimidazolones (X = O). Compound **6a** seems to be the most potent compound in this series, with an IC_{50} of 30 nM in a PR binding assay and an IC_{50} of



5, X = O, S **6,** X = O, S

7, X = O, S **8**

Fig. (5). New non-steroidal progestin receptor ligands. Indolones (**5**), benzimidazolones (**6**), benzoxazinones (**7**) and benzoxazines (**8**).

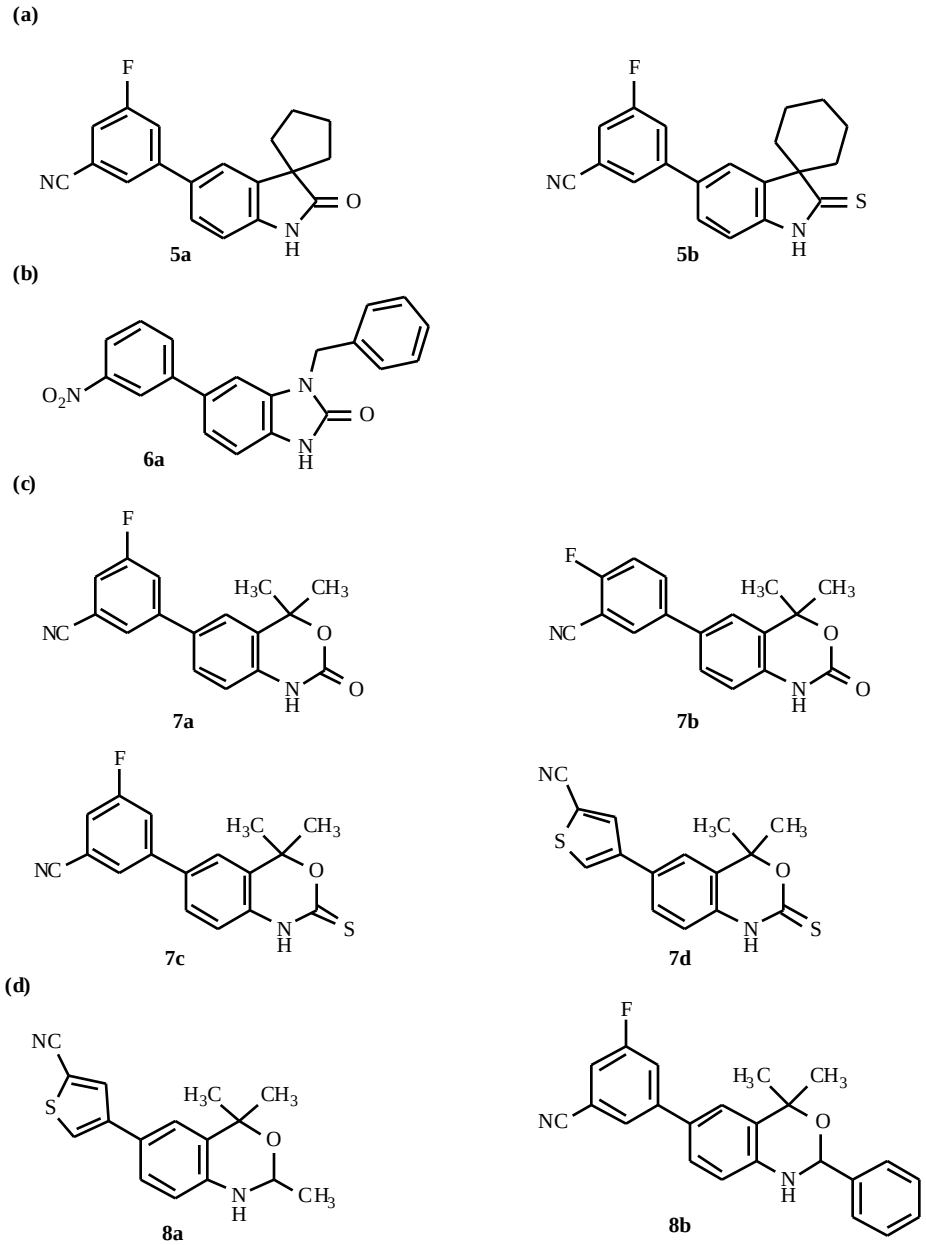


Fig. (6). Indolone, benzimidazolone, benzoxazinone and benzoxazine analogs. (a) Indolones, (b) a benzimidazolone, (c) benzoxazinones, (d) benzoxazines.

10 nM in a T47D cell alkaline phosphatase assay, compared with 1.1 nM and 0.2 nM, respectively, for mifepristone [53].

The benzoxazinones (**7**, X = O) are similar to the indolones in that they display, in general, PR antagonist activity while the benzoxazinethione derivatives (X = S) are potent agonists. It is worth mentioning that some of the benzoxazinones exhibit agonist activities whereas their close analogs are antagonists in the alkaline phosphatase assay in T47D. For example, compound **7a** (Fig. **6c**) is a PR agonist in the T47D functional assay (EC_{50} = 90 nM), while **7b** is an antagonist in the same assay with an IC_{50} of 15 nM. Both compounds were antagonists in a CV-1 cell luciferase reporter gene assay with IC_{50}s of 16 nM and 55 nM, respectively, and were potent antagonists (ED_{50} = 0.3 or 1.0 mg/kg and 0.6 or 4.8 mg/kg in decidualization and C3 assays, respectively) in rats by the oral route [54]. The benzoxazine-2-thiones, such as **7c** and **7d,** were sub-nanomolar PR agonists in the alkaline phosphatase assay (EC_{50} = 0.6 nM and 0.38 nM, respectively). Both compounds were potent PR agonists in the rat decidualization assay by the oral route (ED_{50} = 0.40 mg/kg and 0.62 mg/kg, respectively). The compounds also demonstrated good selectivity for PR over other steroid receptors, such as the androgen and glucocorticoid receptors [55].

The benzoxazines (**8**) have also been reported to be potent PR modulators, with binding affinities to the receptor in the nanomolar range [56]. Many of these analogs exhibited potent PR agonist activity in alkaline phosphatase assays; *e.g.* **8a** (EC_{50} = 0.35 nM) (Fig. **6d**). Based on a limited number of compounds, a large substitution at the 2-position

seemed to convert the compounds to PR antagonists; *e.g.* **8b** (IC_{50} = 93 nM).

CLINICAL APPLICATIONS

Steroidal progesterone receptor ligands have long been used for contraception and hormone replacement, most often in combination with an estrogen. Non-steroidal progestins will likely serve the same uses. In addition, the predicted improved therapeutic ratio of non-steroidal compounds should allow the development of effective therapies for a number of reproductive disorders previously envisioned to be amenable to intervention with progestin receptor ligands. These include endometriosis, uterine leiomyoma, dysfunctional uterine bleeding, and cervical dilation. Whether a given compound is an agonist or an antagonist of progesterone in a given tissue will determine the types of therapy for which it is most appropriate. And with the on-going and future development of selective progesterone receptor modulators (PRMs), it is likely that more specific and effective therapeutic possibilities will emerge over time. For comprehensive reviews of the pharmaceutical applications of progestin receptor ligands, see references [3,6,57,58].

Contraception

Progesterone is required for the establishment and maintenance of pregnancy, and so the disruption of its normal functioning is an effective method of contraception. Administration of a progestin alone disrupts the hypothalamic-gonadal signaling axis, interfering with ovulation. In addition, endometrial implantation is prevented. In most contraceptive regimens, an estrogen and a progestin are given in combination, providing the safest and most tolerable effect. Although not yet widely used in cyclic regimens, progestin antagonists are also effective contraceptives, as they can both inhibit ovulation and endometrial proliferation. Mifepristone is a safe and effective emergency contraceptive.

To our knowledge, the most advanced non-steroidal progestin in clinical development is WAY-166989, co-developed by Wyeth and Ligand Pharmaceuticals. It is being tested for use in contraception and hormone replacement therapy, and, if approved, would be the first non-steroidal progestin receptor ligand to be widely used in humans. It appears to be either an indolone or benzoxazinone analog (Fig. **6**).

Hormone Replacement Therapy

Estrogen has routinely been given to post-menopausal women to alleviate some of the symptoms of estrogen loss, and to prevent excessive loss of bone. As in contraceptive regimens, progestins are often given with the estrogen to counteract the proliferative effects of the latter on the uterine endometrium. Other benefits were expected, including a reduction in cardiovascular disease and memory loss. However, a prospective trial of over 16,000 post-menopausal women (the Women's Health Initiative) has shown that a widely used estrogen plus progestin combination actually increased the risks of coronary heart disease, breast cancer, stroke and pulmonary embolism, while reducing the risks of endometrial cancer and hip fractures [59]. Whether this surprising finding was due to the exact combination used (conjugated estrogens and medroxyprogesterone acetate) remains to be seen. It is also not clear if combinations with new non-steroidal progestins, or with progestin antagonists, will be safer. But it is worth pointing out that another arm of the same study, with estrogen alone, has yet to reveal these increased risks.

Endometriosis and Uterine Leiomyoma

Both endometriosis (characterized by cycling endometrial tissue outside the uterus) and uterine leiomyomata (benign fibrotic growths in the myometrium of the uterus) are hormone-dependent conditions with limited therapeutic options. In small, uncontrolled clinical trials, the steroidal progestin antagonist mifepristone was shown to reduce the pain associated with endometriosis, and to decrease the sizes of lesions [60]. It may do this by inhibiting estrogen-dependent endometrial proliferation, although the molecular mechanism underlying the effect is not fully understood [58]. Likewise, some clinical data indicate that mifepristone can decrease the size of fibroids [61], probably by antagonizing a stimulatory effect of progesterone on myoma growth [62]. Non-steroidal progestin antagonists may prove useful to treat these conditions.

Other Indications

As already mentioned, mifepristone can inhibit endometrial proliferation. In monkeys, this is accompanied by amenorrhea [63]. For this reason, steroidal and non-steroidal progestin antagonists could be useful for abnormal bleeding during the menstrual cycle, though no clinical studies have yet tested the possibility. Finally, progestin antagonists can dilate and soften the uterine cervix, possibly by preventing progestogenic inhibition of nitric oxide production [64]. While mifepristone was initially promising as an agent for labor induction, several recent trials have shown minimal clinical advantage of the compound over placebo [65].

CONCLUSIONS

This is an interesting time for those who work on the progesterone receptor and its ligands. This is because, first of all, genetic analyses in mice have revealed a lot of information about the biology of the receptor, in particular on the separate and interactive roles of the two isoforms in different tissues. Further examination of the physiology of the various null mutant and transgenic models, and future development of tissue-specific knockouts of the receptor, will add to our understanding of progesterone receptor biology. Of major interest will be the role of the receptor isoforms in mammary proliferation and tumorigenesis. Secondly, new, non-steroidal progestin receptor ligands are in clinical trials, and may reach the market in the next few years. These compounds will provide a new option for current contraceptive and hormone replacement regimens, and should stimulate new clinical research into gynecological diseases with unmet medical needs, such as endometriosis, uterine leiomyomata and dysfunctional uterine bleeding.

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