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Specific interactions of steroids, arylhydrocarbons and flavonoids with progesterone receptors from the cytosol of the fungus *Rhizopus nigricans*

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Abstract

Rhizopus nigricans (*R. nigricans*) transforms fungitoxic progesterone into the less toxic 11α-hydroxyprogesterone which is then able to exit the mycelia into the surrounding water. Hydroxylation of progesterone is an inducible process in which cytosolic progesterone receptors could be involved. In the present study, we characterised receptors with respect to ligand specificity and to their involvement in progesterone induction of hydroxylase. EC_{50} values of different ligands (steroids, xenobiotic arylhydrocarbons and natural flavonoids) were determined by competition studies using 40 nM (^{3}H)progesterone. C21 and C19 3-oxo-4-ene steroids were good competitors (EC_{50} of progesterone $2.3 \pm 0.1 \times 10^{-7}$ M, EC_{50} of androsten-3,17-dione $24 \pm 2 \times 10^{-7}$ M). The presence of hydroxyl groups in steroids significantly decreased the affinity for receptors. The arylhydrocarbons α-naphthoflavone and ketoconazole exhibited EC_{50} values of $0.3 \pm 0.01 \times 10^{-7}$ M and $27 \pm 5 \times 10^{-7}$ M, respectively, whereas β-naphthoflavone and benzo(a)pyrene were not able to displace labelled progesterone completely. The competition curves obtained by natural flavonoids also did not reach the bottom level of non-labelled progesterone, indicating the interaction at some allosteric binding site(s) of progesterone receptors. All ligands were examined for their involvement in progesterone-hydroxylase induction. Steroid agonists induced the enzyme in a dose-dependent manner in accordance with their affinity for receptors, whereas arylhydrocarbons and natural flavonoids did not induce the enzyme. The agonistic action of steroids, together with the antagonistic action of α-naphthoflavone, strongly suggests the involvement of progesterone receptors in progesterone signalling resulting in the induction of progesterone-hydroxylase.
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Keywords: *Rhizopus nigricans*; Progesterone hydroxylation; Progesterone receptor; Steroids; Arylhydrocarbons; Flavonoids

1. Introduction

Rhizopus nigricans (*R. nigricans*), a saprophytic fungus from the phylum *Zygomycetes*, is a widespread microorganism, found in the soil, in gathered crops (grain, vegetables) as well as in manmade substrates such as flour and bread. Like other fungi, *R. nigricans* is able to respond to signals from the surroundings (such as xenobiotics and antifungal compounds) by adapting its metabolism to different environmental conditions. *R. nigricans* can also be grown successfully in artificial growth medium, where it is possible to aim fungal metabolism to a desirable product. When the fungus is exposed to fungitoxic progesterone the latter is converted predominantly into the 11α-hydroxy derivative [1], an important intermediate in the industrial production of corticosteroids [2]. The purpose of this hydroxylation is, most likely, detoxification of xenobiotic progesterone, rendering it more water soluble and thus more easily removable from mycelia into the surrounding water [3]. It was established that progesterone-hydroxylase in *R. nigricans* is a cytochrome P450-dependent enzyme system [4,5]. These enzymes are generally involved in oxidative conversion of xenobiotics into entities which can be readily excreted and detoxified [6–8].

In *R. nigricans*, progesterone-hydroxylase is inducible by progesterone and some other steroid inducers [9,10]. The mechanism of enzyme induction by progesterone has not yet been elucidated, although some similarities to steroid action

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in higher eucaryotic organisms were observed. In our preliminary reports, we detected progesterone binding molecules in the *R. nigricans* plasma membrane fraction [11] and in the cytosol [12]. No biological role of membrane receptors in progesterone signalling has been detected; we were not able either to confirm or to exclude the participation of cytosolic receptors in the induction of progesterone hydroxylases [12]. By finding an antagonist of cytosolic progesterone receptors it should be possible to establish the involvement of these binders in the induction process. In the present study, we performed structure-affinity experiments of putative receptor ligands using different steroids, xenobiotic arylhydrocarbons and flavonoids. The mode of action of arylhydrocarbons in mammalian cells is known to be via ligand activated transcription factors, similar to that of steroids [13,14] and they are involved in detoxification processes [15,16]. Flavonoids, on the other hand, are components of the plant defence system [17,18] and have been shown to interfere with steroid signalling in higher organisms [19,20].

2. Experimental

2.1. Chemicals

Chemicals were obtained from the following companies: [1,2,6,7-^{3}H]progesterone – 81 Ci/mmol from NEN Research Products (Boston, MA); onapristone from Scherring (Berlin, Germany); corticosterone from Steraloids (Newport, RI); other unlabelled steroids, α- and β-naphthoflavone, ketoconazole, benzo(a)pyrene, metyrapone, griseofulvin, tomatidine and genistein from Sigma Chemical Co. (St. Louis, MO); chrysin, apigenin, and kaempferol from Extrasynthese (Genay, France); methylcholanthrene from Hoffmann-La Roche (Basle, Switzerland); Sephadex LH-20 from Pharmacia (Uppsala, Sweden). 24-Epicastasterone was kindly provided by Dr. Ladislav Kohout, Institute of Organic Chemistry and Biochemistry, Academy of Sciences, Czech Republic.

All other reagents were of analytical grade and were prepared in deionized water.

2.2. Micro-organism

Filamentous fungus *R. nigricans* ATCC 6227 from the phylum *Zygomycetes*, obtained from MZKI (Ljubljana, Slovenia) was grown for 18 h at 28 °C as described [9].

2.3. Progesterone hydroxylating enzyme induction assay

The ability of several compounds to induce progesterone-hydroxylase was examined after cultivating the fungus for 18 h. Compounds were introduced into the growth medium in final concentrations presented in Results. The fungus was cultivated for an additional 2 h, followed by steroid hydroxylase assay, using progesterone (300 μM) as a substrate. The enzyme reaction proceeded for an additional 30 min and was terminated by washing off the growth medium and extracting the fungus with chloroform. Chloroform extracts were analysed by TLC and the yield of 11α-hydroxyprogesterone evaluated densitometrically [4]. When it was not possible to separate compounds of similar hydrophobicity (e.g. progesterone and naphthoflavone) by TLC, the separation was achieved by HPLC using a Hypersil C-18 column (15 cm).

The effect of different compounds on enzyme induction by progesterone was examined by simultaneous addition of these compounds and progesterone into the medium followed by the standard enzyme assay. In parallel experiments, the possible direct interaction of inhibitors with the cytochrome P450 system was examined. After 2 h of hydroxylase induction by progesterone, the enzyme was assayed in the presence of inhibitor (0.1, 1.0 and 10-fold excess over the substrate progesterone).

2.4. Homogenisation procedure and preparation of cytosol

Fungal mycelia were homogenised in TEGMP buffer (10 mM Tris–HCl, 1 mM EDTA, 10% glycerol, 2 mM monothioglycerol, 1 mM PMSF, pH 7.4), and cytosol was prepared as described [12].

2.5. Competition binding experiments

Steroids and non-steroidal compounds were used as progesterone competitors. Steroids were selected with regard to their structural differences from progesterone. Chemical structures of those used in this study are shown in Fig. 1: (a) 3-oxo-4-ene steroids with modifications at C17 (pregnenes and androstenes) and synthetic progesterone agonist R5020 with partially dehydrogenated B ring; (b) 3-oxo-4-ene steroids with modifications at C11 and some additional modifications at C17 (mammalian progesterone antagonists mifepristone and onapristone); (c) C21 steroids and C19 steroids with reduced A ring (pregnanes and androstanes); (d) steroids with aromatic A ring; (e) C24, C27 and C28 steroids containing C3 OH-group as well as some additional OH-groups and further modifications. Chemical structures of non-steroidal ligands, xenobiotic arylhydrocarbons and natural flavonoids are shown in Fig. 2 and Fig. 3, respectively.

Prior to the competition experiment, cytosol was treated with dextran coated charcoal to remove endogenous substances which could interfere with the binding assay [12]. Samples of the stripped cytosol (100 μl) were incubated with equal volumes of 80 nM (^{3}H)progesterone in TEMGP buffer alone, or with (^{3}H)progesterone in the presence of different excesses of selected competitors for 45 min at 22 °C. Bound and non-bound steroids were separated by Sephadex LH-20 gel exclusion chromatography [12] and bound radioactivity determined in an LKB 1214 Rackbeta liquid scintillation counter. Results were analysed by the Prism 2 computer package (Graphpad, Dan Diego, CA, USA).

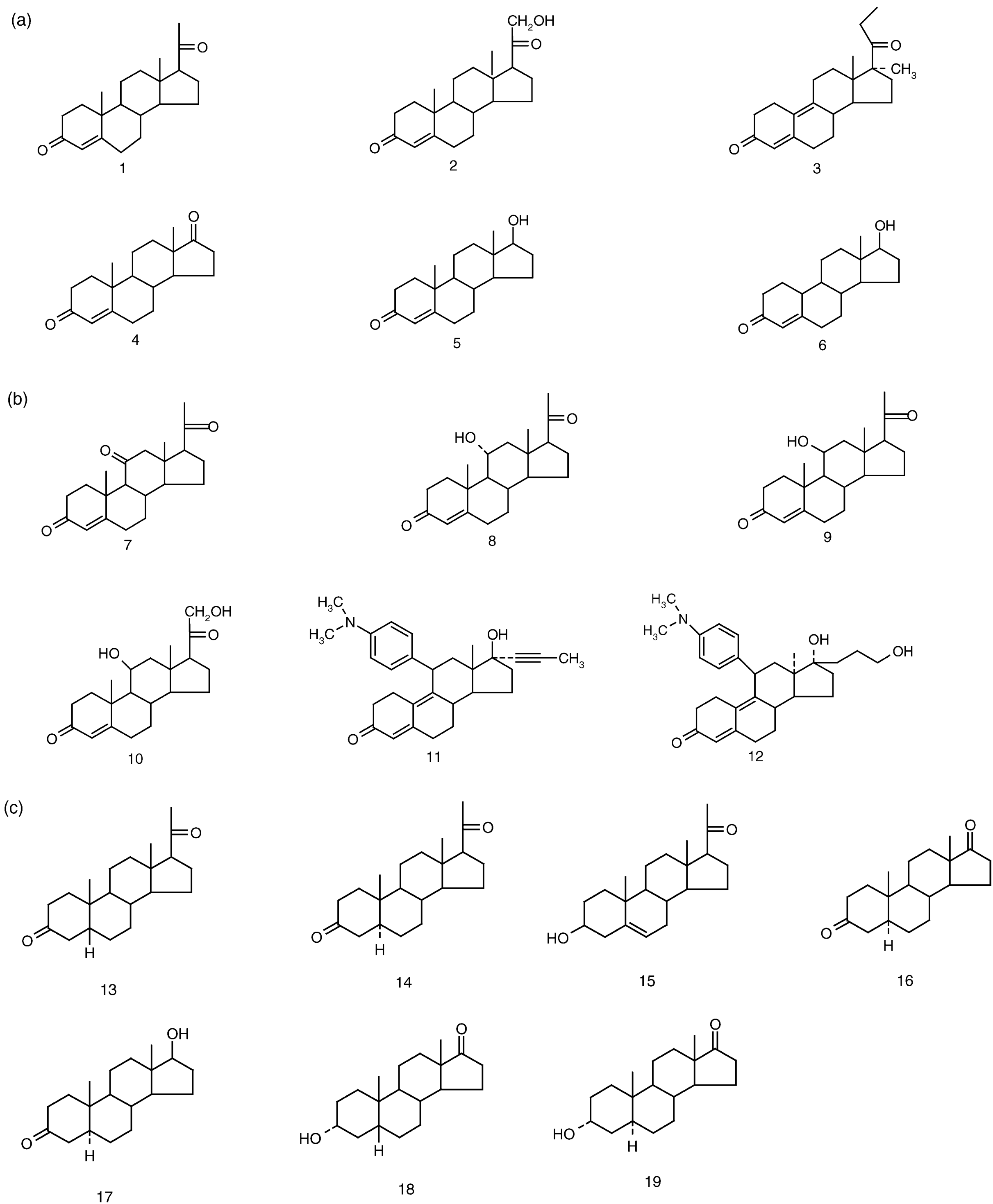

Fig. 1. Chemical structures of steroids: (a) 1, progesterone; 2, 21-hydroxyprogesterone = deoxycorticosterone; 3, R5020; 4, 4-androstene-3,17-dione; 5, testosterone; 6, nor-testerosteone. (b) 7, 11-oxoprogesterone; 8, 11α-hydroxyprogesterone; 9, 11β-hydroxyprogesterone; 10, corticosterone; 11, mifepristone; 12, onapristone. (c) 13, 5β-pregnane-3,20-dione; 14, 5α-pregnane-3,20-dione; 15, pregnenolone; 16, 5α-androstane-3,17-dione; 17, 5α-androstane-17-ol,3-one; 18, etiocholanone; 19, androsterone; (d) 20, U73122; 21, estradiol-17β; 22, ethisterone; (e) 23, egosterol; 24, 24-epicastasterone; 25, tomatidine; 26, cholesterol.

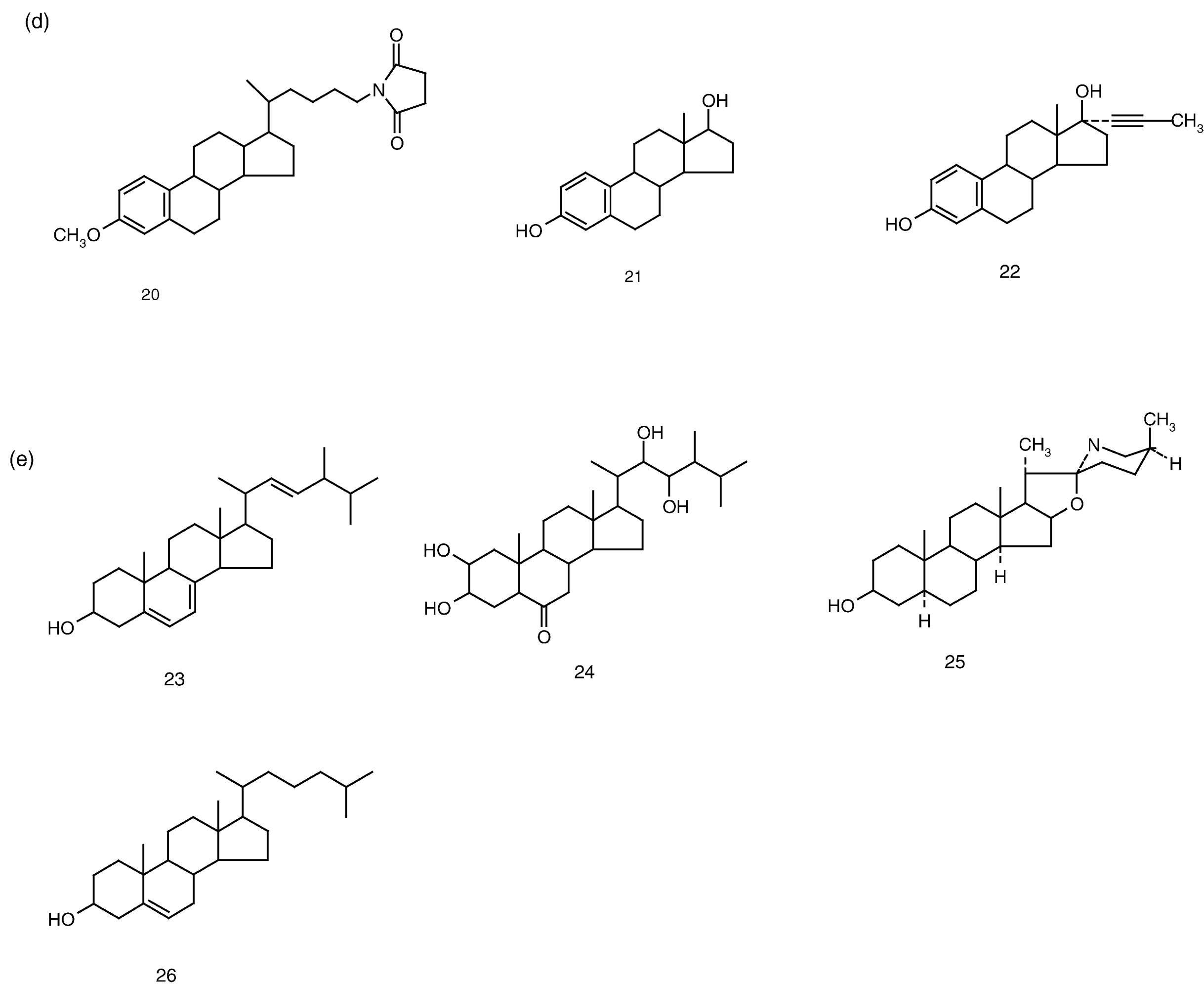

Fig. 1. (*Continued*).

3. Results

3.1. Steroid ligands of progesterone receptors

Displacement of labelled by non-labelled progesterone from cytosolic binding sites was characterised by EC_{50} value of $2.3 \pm 0.1 \times 10^{-7}$ M (the mean ± S.E.M, $n = 22$). For the sake of clarity in graphic presentation of competition ability of selected ligands the displacement by progesterone is presented only at 0.2-, 1-, 10-, 100-, 1000- and 2000-fold excess of non-labelled over the labelled progesterone. Competition experiments using different steroids (Fig. 1) is shown in Fig. 4. The following order of affinities of 3-oxo-4-ene steroids for progesterone receptors was determined (Fig. 4a): progesterone, 4-androsten-3,17dione ($EC_{50} = 24 \pm 2 \times 10^{-7}$ M), 21-hydroxyprogesterone ($EC_{50} = 27 \pm 2 \times 10^{-7}$ M), R5020 ($EC_{50} = 111 \pm 15 \times 10^{-7}$ M), testosterone ($EC_{50} = 131 \pm 17 \times 10^{-7}$ M), whereas 19-nor-testosterone was not able to completely displace labelled progesterone, indicating the importance of the methyl group at C10 in the ligand-receptor interaction. EC_{50} values showed that progesterone exhibited the highest affinity for receptors; any modification, such as introduction of a hydroxyl group into the side chain or elimination of the side chain from C17 led to lower affinity. An effective progesterone agonist of mammalian progesterone receptor, R5020, was not efficient in binding to fungal progesterone receptor.

C11 progesterone derivatives (Fig. 1b) competed less strongly than progesterone (Fig. 4b): 11-oxoprogesterone ($EC_{50} = 9 \pm 0.6 \times 10^{-7}$ M), 11β-hydroxyprogesterone ($EC_{50} = 80 \pm 12 \times 10^{-7}$ M), 11α-hydroxyprogesterone ($EC_{50} = 430 \pm 52 \times 10^{-7}$ M). Mammalian progesterone antagonists mifepristone and onapristone, with a bulky group at the C11 β-position, competed only weakly (mifepristone – $EC_{50} = 458 \pm 63 \times 10^{-7}$ M) or not at all (EC_{50} of onapristone was not determinable). Corticosterone, with an additional C21 hydroxyl group, bound weakly to the receptors ($EC_{50} = 438 \pm 41 \times 10^{-7}$ M). Comparison of EC_{50} values of steroids containing hydroxyl groups at different positions indicates the greatest disturbing influence of the C11α-hydroxyl, followed by C11β-hydroxyl and C21-hydroxyl group of progesterone derivatives.

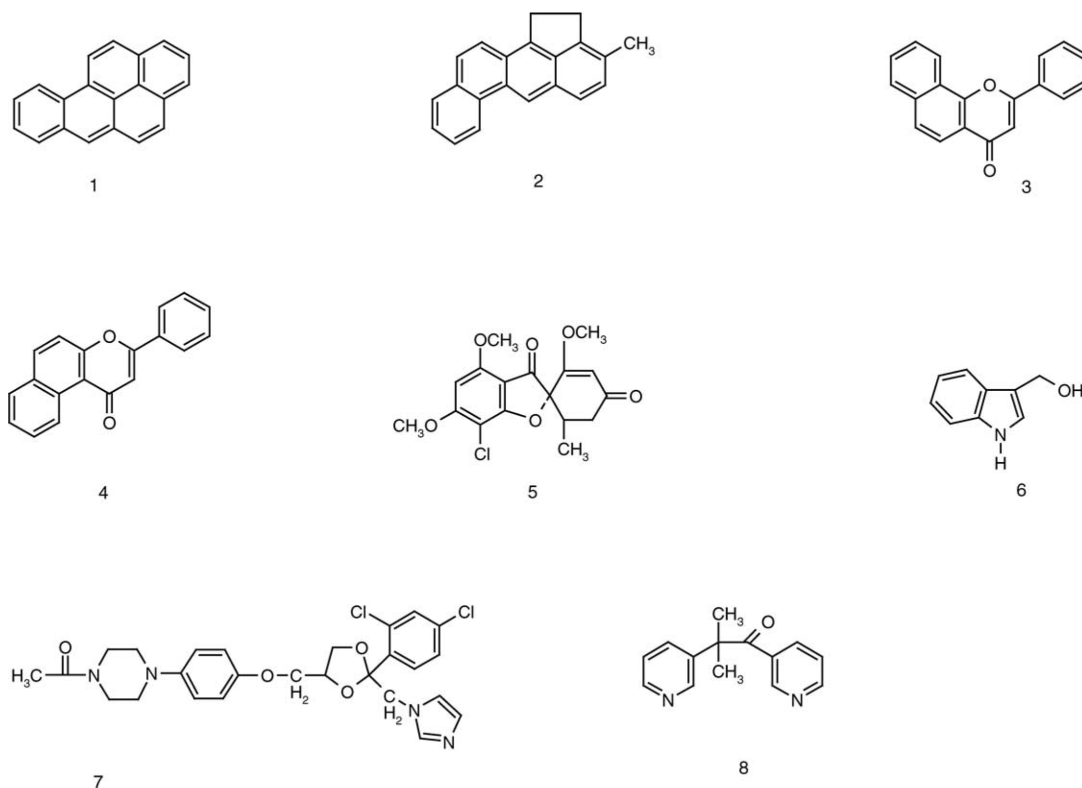

Fig. 2. Chemical structures of selected fungitoxic compounds: 1, benzo(a)pyrene; 2, 3-methylcholantrene; 3, α-naphthoflavone; 4, β-naphthoflavone; 5, griseofulvin; 6, indole-3-carbinol; 7, ketoconazole; 8, metyrapone.

Competition curves of some pregnanes and androstanes (Fig. 1c) are shown in Fig. 4c. EC_{50} values were as follows: 5β-pregnane-3,20-dione (Fig. 1c, 13), $1.5 \pm 0.1 \times 10^{-7}$ M, but because of incomplete displacement by this compound the EC_{50} should be interpreted with caution; 5α-pregnane-3,20-dione (Fig. 1c, 14), $13 \pm 0.9 \times 10^{-7}$ M; pregnenolone (Fig. 1c, 15), $102 \pm 10 \times 10^{-7}$ M; 5α-androstane-3,17-dione (Fig. 1c, 16), $33 \pm 4 \times 10^{-7}$ M. 5α-androstane-17-ol-3-one (Fig. 1c, 17) competed weakly, whereas etiocholanone (Fig. 1c, 18) and androsterone (Fig. 1c, 19) did not compete with progesterone for receptors. By comparing these EC_{50} values, some structural characteristics of pregnanes and androstanes important in competing with progesterone can be identified. (i) In pregnanes, the trans position of rings A and B is important (Fig. 1c, 13 versus 14); the C3 β-hydroxyl group (Fig. 1c, 15) seriously interferes with binding of the

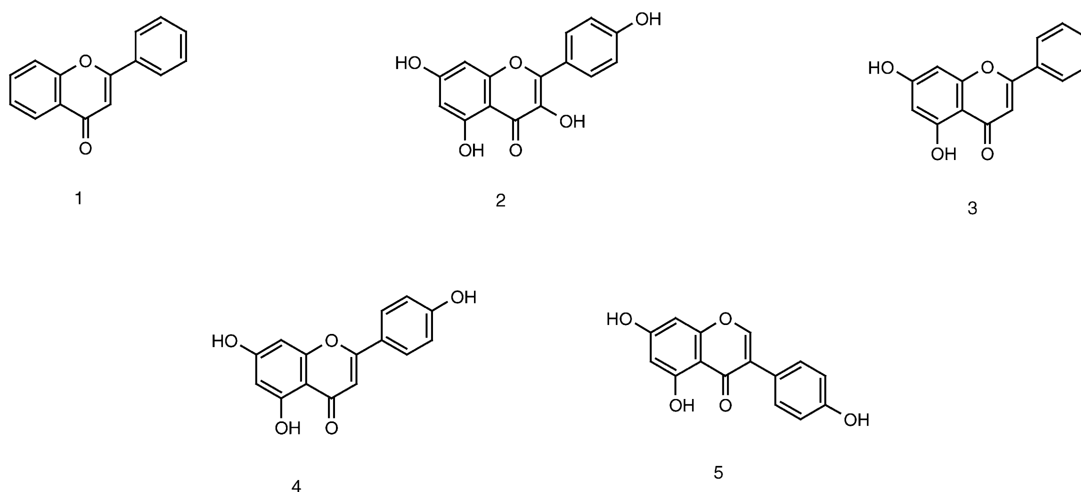

Fig. 3. Chemical structures of selected flavonoids: 1, flavone; 2, kaempferol; 3, chrysin; 4, apigenin; 5, isoflavone genistein.

steroid to receptor. (ii) In androstanes, replacing the C17-oxo group by a hydroxyl group (Fig. 1c, 17 versus 16) reduces the affinity for receptors; a C3 α-hydroxyl group (Fig. 1c, 18, 19) completely eliminates the ability of these compounds to bind to the receptors.

Among steroids with the aromatic A ring (Fig. 1d), only aminosteroid U73122 (Fig. 1d, 20) competed effectively (EC_{50} of $13 \pm 2 \times 10^{-7}$ M) with progesterone. The low receptor affinities of estradiol-17β (Fig. 1d, 21) and ethisterone (Fig. 1d, 22) can most probably be attributed to the C3 hydroxyl group and not to aromatisation of the A ring and modifications at C17.

Steroids of higher M_r are shown in Fig. 1e. Ergosterol competed slightly but the EC_{50} value could not be determined because of uncomplete displacement of labelled progesterone at 10^{-4} M concentration of competitor. No other compounds from this group competed with progesterone for receptors. Because all these steroids contain a C3 OH-group in the re-

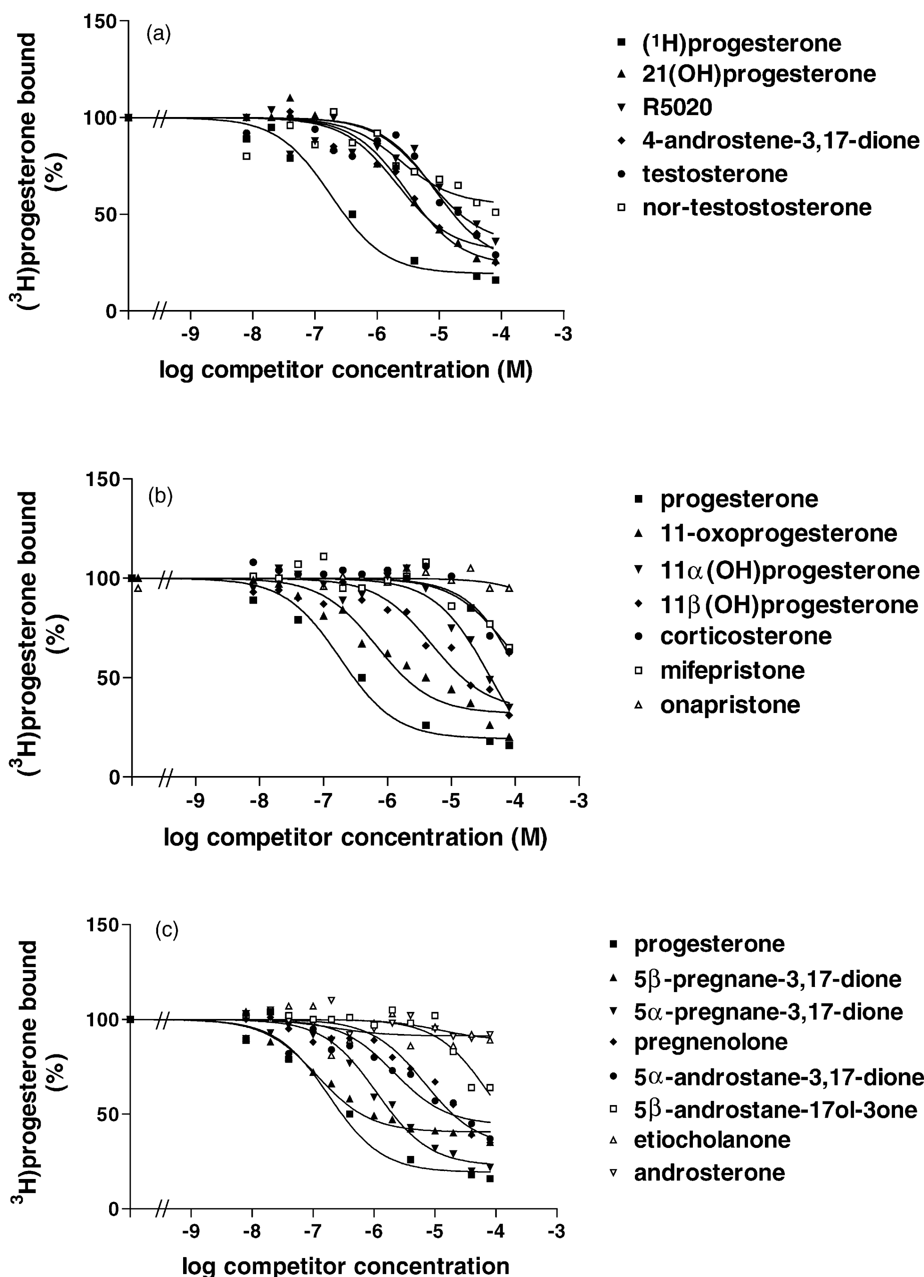

Fig. 4. Competition of steroids for progesterone receptors in *R. nigricans* cytosol. Cytosolic receptors were incubated with 40 nM (^{3}H)progesterone and, in parallel samples, with 40 nM (^{3}H)progesterone in the presence of different excesses of competitors as indicated. Unbound steroids were removed as described in the experimental section. (a–e), displacement of labelled progesterone by steroids presented in Fig. 1(a–e), respectively. Results were analysed by the Prism programme – one site competition equation. Each point represents the mean value of three independent determinations. Standard errors of the mean never exceeded 15% and are omitted for clarity.

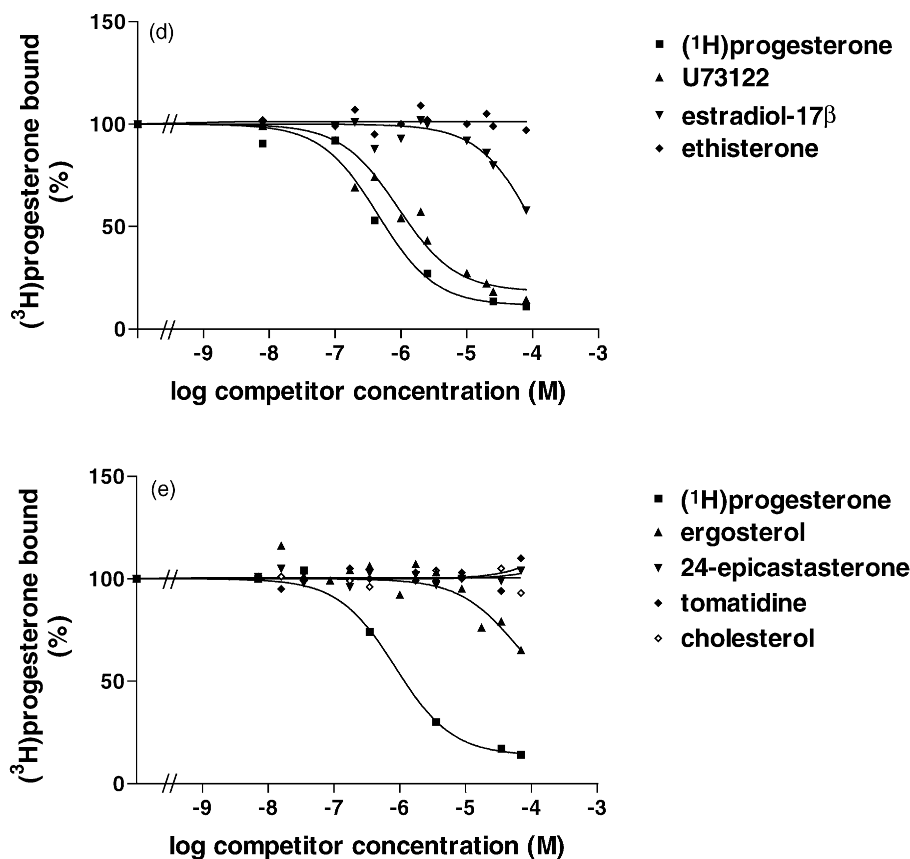

Fig. 4. (*Continued*).

duced A-ring, their low binding affinity is in accordance with results presented above (Fig. 4c and Fig. 4d). Elongated side chains with additional modifications, e.g. 24-epicastasterone with multiple hydroxyl groups, cholesterol and tomatidine (Fig. 1e), caused complete loss of binding affinity for progesterone receptors.

We can conclude that progesterone (3-oxo-4-pregnene) is the most effective ligand of *R. nigricans* cytosolic receptors followed by 3-oxo-5α-pregnanes without OH-groups at C11 or in the side chain at C17.

3.2. Non-steroid ligands of progesterone receptors

In a search for possible non-steroidal ligands of *R. nigricans* progesterone receptor we examined some aryl-compounds (Fig. 2) which are known to regulate the expression/activation of cytochromes P450 in mammals [15,16], invertebrates [8,21] and fungi [22,23]. In addition, we tested some flavonoids (Fig. 3) which are involved in plant defence processes [17].

Of the fungitoxic compounds, α-naphthoflavone and ketoconazole were good competitors of progesterone, exhibiting EC_{50} values of $0.3 \pm 0.02 \times 10^{-7}$ M and $26.6 \pm 3 \times 10^{-7}$ M, respectively; the former was more effective than progesterone itself (Fig. 5). β-naphthoflavone, benzo(a)pyrene and metyrapone (an inhibitor of 11α-progesterone-hydroxylase in *R. nigricans* [3]) were better competitors than progesterone up to a 5-fold excess over labelled progesterone but not at higher concentrations (Fig. 5). Similar incomplete displacement was achieved by 3-methylcholanthrene, but this compound acted only as a weak competitor. Griseofulvine (antifungal antibiotic) and indole-3-carbinol (precursor of indolo(2,3-b)carbazole, an efficient ligand of arylhydrocarbon receptor [24]), did not compete with progesterone for its receptors (Fig. 5).

All the natural flavonoids tested (flavones: flavone, chrysin, apigenin; flavonol: kaempferol; isoflavone: genistein) were able to recognise fungal progesterone receptors but their competition curves did not reach the level of non-labelled progesterone (Fig. 6). The most effective in this activity was chrysin, followed by flavone, kaempferol, and apigenin, but the isoflavone genistein exhibited only a marginal effect on progesterone binding to receptors.

3.3. Induction of 11α-progesterone-hydroxylating enzymes by receptor ligands

With the aim of assessing the involvement of progesterone receptors in the induction of progesterone-hydroxylase, we tested some receptor ligands for their ability to induce the enzyme. Good progesterone competitors (progesterone, 21(OH)progesterone, moderate competitor (testosterone)

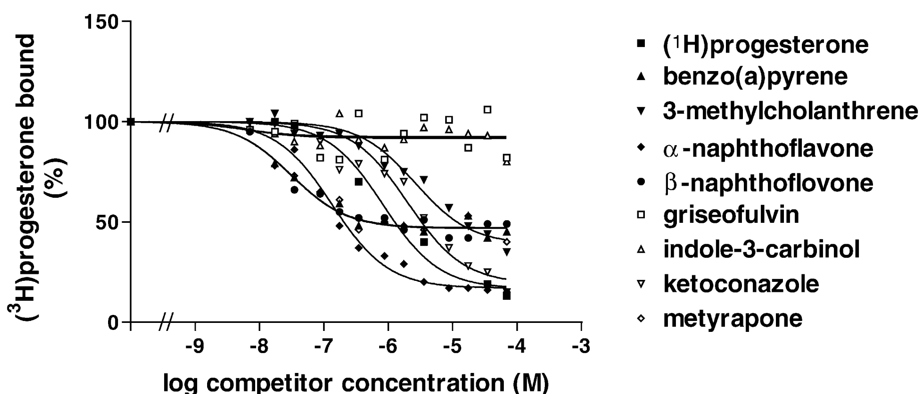

Fig. 5. Competition for progesterone receptors in *R. nigricans* cytosol by fungitoxic compounds shown in Fig. 3. The experimental procedure and the analysis of results is explained in the legend to Fig. 4.

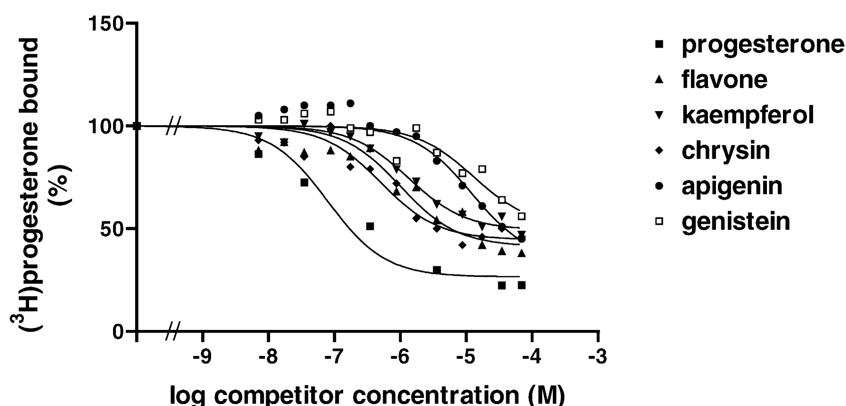

Fig. 6. Competition for progesterone receptors in *R. nigricans* cytosol by flavonoids shown in Fig. 3. The experimental procedure and the analysis of results is explained in the legend to Fig. 4.

and weak competitor (estradiol-17β) were chosen. Results are shown in Fig. 7. Progesterone, 21(OH)progesterone and testosterone exhibited a clear dose-dependent induction of hydroxylase up to 30 μM concentration, whereas estradiol-17β showed no activity. The ability of the steroids to induce hydroxylase correlates well with their affinity for cytosolic receptors (Fig. 4a and c).

Non-steroidal ligands which exhibited high affinity (arylhydrocarbons, Fig. 5) and moderate affinity (flavonoids, Fig. 6) for progesterone receptors were also examined for their ability to induce progesterone-hydroxylase. Neither arylhydrocarbons (ketoconazole) nor synthetic (α- and β-naphthoflavone) or natural flavonoids (apigenin, chrysin and kaempferol) showed any inducing activity. Herewith the antagonistic action of α-naphthoflavone, a ligand with the highest affinity for progesterone receptors, was revealed.

We also examined the effect of those ligands for the receptors, which were noninducers, on the induction of enzyme by progesterone. A clear dose-dependent effect of α-naphthoflavone was observed (Fig. 8b). A direct effect of α-naphthoflavone on cytochrome P450 was excluded by measuring the P450 enzyme activity in the presence of α-naphthoflavone (0.1–10-fold excess over substrate pro-

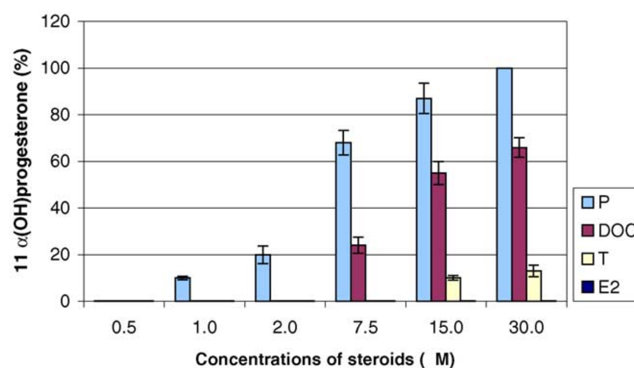

Fig. 7. Dose dependence of progesterone-hydroxylase induction by steroids. After growing for 18 h *R. nigricans* was incubated for an additional 2 h with steroids (P, progesterone; DOC, deoxycorticosterone = 21-hydroxyprogesterone; T, testosterone; E2, estradiol-17β) at final concentrations as indicated. Subsequently, hydroxylation activity was assayed using progesterone as substrate [9]. Results are presented in percent of conversion of progesterone into 11α-hydroxyprogesterone, where the maximal conversion obtained by 30 μM progesterone was defined as 100%. All values are the mean ± S.E.M. ($n = 3$).

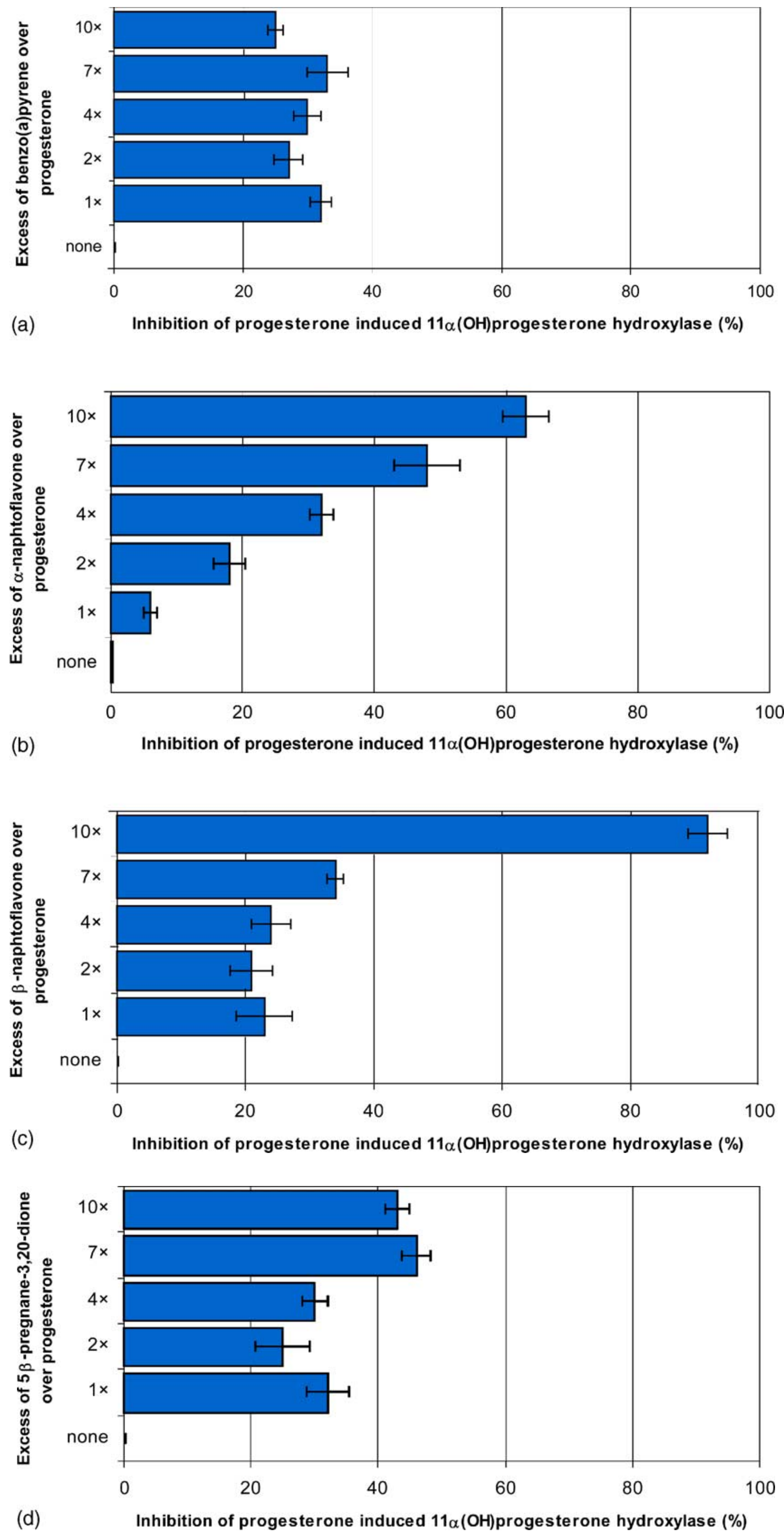

Fig. 8. Effect of selected progesterone receptor ligands on progesterone-hydroxylase induction by progesterone. *R. nigricans* was grown for 18 h and for an additional 2 h with 15 μM progesterone alone or with simultaneous addition of indicated excesses of benzo(a)pyrene (a), α-naphthoflavone (b), β-naphthoflavone (c) and 5β-pregnane-3,20-dione (d). Hydroxylation activity was assayed using progesterone as a substrate and the yield of 11α-hydroxyprogesterone determined. Results are presented in percent of inhibition of enzyme induction by progesterone where 0 represents the induction without inhibitors and 100% represents completely inhibited induction by progesterone. All values are the mean ± S.E.M. ($n = 3$).

gesterone) – no inhibition was observed. Enzyme induction by progesterone was also inhibited by β-naphthoflavone, benzo(a)pyrene and 5β-pregnane-3,17-dione, but not in a dose-responsive manner (Fig. 8a, c, d). This result suggests some additional interaction site(s) on the progesterone receptor molecule which, when occupied by an allosteric modulator, undergoes a conformational change resulting in modification of the principal progesterone binding site.

4. Discussion

R. nigricans responds to xenobiotic progesterone from the surroundings by inducing progesterone-hydroxylating enzymes containing cytochrome P450 [4,10]. During our investigation of progesterone signalling in the fungus two different routes were proposed, one initiated by progesterone binding to receptors at the fungal plasma membrane [11] and the other involving intracellular progesterone receptors [12]. Our previous studies were focused on the kinetic characteristics of progesterone receptors, resulting in identification of a single type of membrane receptor with a K_d of ≈50 nM [11] and two types of cytosolic receptors with K_d of ≈5.0 nM and ≈30.0 nM [12]. In the present investigation we focused on cytosolic progesterone receptors and their involvement in progesterone-hydroxylase induction together with their ligand specificity. The results (Fig. 7) strongly suggest the involvement of cytosolic receptors in the induction process, since ligands with high affinity for receptors (progesterone, 21(OH)progesterone, Fig. 4a) were good inducers, in contrast to one with low affinity (estradiol-17β, Fig. 4d) which did not induce progesterone-hydroxylases. The high affinity progesterone receptors ($K_d \approx 5.0$ nM) are most probably not suitable for progesterone signalling in detoxification because their low capacity is not sufficient to mediate the transformation of the abundant progesterone in the surroundings. The role of these receptors might be to regulate fungal physiology by some unknown endogenous compound; so far we have not been able to identify a putative endogenous ligand. On the other hand, low affinity progesterone binders ($K_d \approx 30$ nM) seem more appropriate to be involved in the detoxification of the relatively high amount of progesterone from the medium. The apparent discrepancy between inducing progesterone in the μM concentration range and the affinity for binding sites (K_d of 30 nM) could be explained by the experimental conditions. Induction was studied in vivo, where progesterone had to pass the cell wall as well as the plasma membrane to reach responding binding sites inside the mycelia, whereas the affinity of receptors for progesterone was examined in the isolated cytosolic fraction. A similar observation was reported by Clemons et al. [25] who, in the cytosol of pathogenic fungus *Trychophyton mentagrophytes,* observed a 100-fold higher affinity for progesterone binding sites (K_d of 95 nM) when compared to the environmental progesterone concentration which inhibited fungal growth by 50% at 5.5 μM. This discrepancy was ascribed to impeded entry of progesterone into intact cells.

Based on the affinity of fungal receptors for progesterone we performed competition studies using 40 nM (^{3}H)progesterone. Different steroids exhibited relatively high affinities for receptors (Fig. 4) but the best steroidal ligand was progesterone with its nearly planar steroid ring structure and 4-ene,3-one characteristics. Any modification of the progesterone molecule, such as C4–5 reduction and 11α-, 11β- and 21-hydroxylation, led to lowered affinity for receptors. An exception was 5β-reduction of the double bond, resulting in 5β-pregnane-3,20-dione which was not able to completely displace labelled progesterone (Fig. 4c), possibly because of its bent nonplanar structure. Interaction of the fungal receptor with steroids appears to be through the α-face, since the OH-group at C11-α constitutes a greater hindrance than that at C11-β (the lack of affinity for progesterone receptors of mammalian antagonists might be explained by inconveniently bulky groups at the C11β-position). This result is in accordance with the interaction of the mammalian genomic nuclear receptor via the α-face of progesterone [26]. The biological significance of the decreased affinity of fungal progesterone receptors for 11α-hydroxyprogesterone appears logical, since progesterone acts as an inducer of its own transformation into the 11α-hydroxy-product [1] which is eliminated from mycelia [3].

The steroid specificity of *R. nigricans* progesterone receptors is in accordance with results of Nobel et al. [27] who stressed the importance of metabolic conversion of steroids as a pre-receptor mechanism of control of steroid hormone action in mammals. Only steroids displaying distinct structural properties act as ligands of progesterone receptors; the nearly planar steroid ring structure and the 3-oxo configuration of progesterone is necessary for successful association with receptor. Metabolic transformation of these chemical configurations therefore has a profound effect on receptor binding, e.g. reduction of the 3-oxo group to 3α- or 3β-hydroxyls. Other different hydroxylations, as well as loss of the planar structure by 5β reduction of the C4-5 double bond, lead to reduced or complete loss of binding properties [27].

We compared fungal receptor steroid specificity with the results of Mais et al. [28] who studied specific interactions of progestins and anti-progestins with progesterone antibodies, plasma binding proteins and human recombinant receptor. The fungal receptor specificity was identical to the ligand specificity of mammalian plasma progesterone binding proteins. Bearing in mind the biological role of plasma binding proteins in mammals one can speculate that, besides the receptor role in regulation of hydroxylase gene expression in *R. nigricans*, the function of progesterone binders is to withhold the toxic progesterone until there exists the possibility for its transformation by enzymes. Fungal progesterone receptors displayed, however, about 100-fold lower affinity for progesterone than mammalian progesterone-binding proteins [28] which is consistent with their putative role in detoxification of large amounts of fungi-toxic steroid from the environment.

In addition to pregnane derivatives we examined the affinity for *R. nigricans* cytosolic receptors of some hydroxylated higher steroids, derivatives of estrogen and androstane (Fig. 1). From sterols, only the fungal membrane component ergosterol competed slightly for receptors whereas other steroids in this group were not recognised by progesterone receptors (Fig. 4e). Apart from the general hydrophilic character of these compounds, the hindrance to receptor binding is due, most probably, to the C3 hydroxyl-group. This disturbing effect was also present in estradiol and ethisterone and was eliminated when the C3 hydroxyl-group was methylated (U74122, Fig. 1d, Fig. 4d). C19 steroids also exhibited lower affinity for progesterone receptors than C21 steroids, especially when a C3-hydroxyl group was present (Fig. 1b, Fig. 4b).

We extended our study of putative *R. nigricans* progesterone receptor ligands to some well known xenobiotic arylhydrocarbons which are potent arylhydrocarbon receptor agonists, inducing CYP1A1 in mammals and fish [21] as well as in a yeast expression system [22]. α-naphthoflavone and ketoconazole acted as good progesterone competitors in *R. nigricans*, the former exhibiting an even higher affinity for progesterone receptors than progesterone itself (Fig. 5). The competition properties of ketoconazole in *R. nigricans* are in accordance with observation of Eil [29] who detected competitive binding of ketoconazole to both sex steroid binding globulin and multiple steroid hormone receptors, suggesting that the ligand binding sites of these proteins share some features in common. On the other hand, Svec [30] reported that antiglucocorticoid ketoconazole was bound to a putative second regulatory site which, through allosteric mechanisms, altered the binding affinity of the agonist site for its preferred steroid. Other competitors tested in *R. nigricans*, benzo(a)pyrene and β-naphthoflavone, were not able to completely displace labelled progesterone (Fig. 5) which might indicate their binding to some binding site on the receptor molecule other than progesterone.

Some natural flavonoids, known to interfere with steroid action via receptors in steroid responsive cells [19,20], were also studied for their binding to *R. nigricans* progesterone receptors. Many flavonoids are also arylhydrocarbon receptor ligands [16,31,32]. All the compounds tested (flavones flavone, chrysin, apigenin, flavonol kaempferol and isoflavone genistein) interfered with progesterone binding to fungal receptors, possibly by binding to an allosteric binding site since their competition curves did not reach the level of competing non-labelled progesterone (Fig. 6). Interestingly, fungal receptors were not able to discriminate between apigenin and genistein, which exhibit progestational and estrogenic steroidal activities, respectively, in mammalian cells [20].

At present, the biological significance of arylhydrocarbon and flavonoid binding to fungal progesterone receptor is not known; in contrast to steroid agonists of progesterone receptors, benzo(a)pyrene, ketoconazole, α- and β-naphthoflavone and natural flavonoids did not act as inducers of progesterone-hydroxylase (results not shown). Moreover, ligands of high affinity for receptors were effective inhibitors of enzyme induction by progesterone (Fig. 8). Strong inhibition, together with high competition ability for progesterone receptors of α-naphthoflavone, strongly indicates the antagonistic character of this compound. The lack of effect of α-naphthoflavone on cytochrome P450 activity which was observed in different mammalian cells ([33] and references therein) confirmed the antagonistic behaviour of α-naphthoflavone at the level of cytosolic progesterone receptors in *R. nigricans*, thus additionally confirming their role in progesterone-hydroxylase induction.

An interesting difference in the inhibition of enzyme induction by progesterone between α-naphthoflavone on the one hand and β-naphthoflavone, benzo(a)pyrene and 5β-pregnane-3,20-dione on the other was observed (Fig. 8). α-naphthoflavone inhibited enzyme induction in a dose-responsive manner, whereas the inhibition by β-naphthoflavone, benzo(a)pyrene and 5β-pregnane-3,20-dione was independent of concentration over a range of 1–10-fold excess over the inducer progesterone. The reason for these two types of inhibition by progesterone might be the different ligand interactions with receptor reflected in different competition curves (Fig. 4c). The exceptional inhibitory action of β-naphthoflavone (10-fold excess over the inducing progesterone) cannot be explained at present. We can only speculate that 150 μM β-naphthoflavone, in addition to its action at the receptor level, might exert some other effects.

In conclusion, *R. nigricans* cytosolic progesterone receptors exhibit a broad steroid specificity and, in addition, they are able to recognise other compounds such as arylhydrocarbons and flavonoids. The interaction of progesterone receptor with agonists results in the induction of progesterone-hydroxylase, in contrast to the interaction with antagonists which also inhibits the induction by progesterone. These results strongly indicate the involvement of cytosolic receptors in the induction of progesterone-hydroxylase by progesterone. The involvement of cytoplasmic progesterone receptors in the induction process does not, however, exclude the participation of the previously detected progesterone signalling pathway initiating at the fungal plasma membrane [11]. According to Wehling [34] and Losel and Wehling [35] non-genomic pathways may exercise some control over the genomic component of the hormonal effect. The possible biological role of the interaction of *R. nigricans* progesterone receptors with non-steroidal ligands is not known at present. Receptor activation by arylhydrocarbons and flavonoids could be involved in the induction of some other putative enzyme system.

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