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Novel Non-Steroidal/Non-Anilide Type Androgen Antagonists with an Isoxazolone Moiety

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Abstract—3-Substituted (*Z*)-4-(4-*N,N*-dialkylaminophenylmethylene)-5(4*H*)-isoxazolones and related compounds were designed and prepared as candidates for structurally novel androgen antagonists. Several compounds showed potent anti-androgenic activity as assessed by nuclear androgen receptor binding assay and growth inhibition assay using androgen-dependent Shionogi carcinoma cells SC-3. They were approximately 10–220 times more potent than flutamide in these assay systems. They also showed anti-androgenic activity toward prostate tumor cell line LNCaP, which has an aberrant nuclear androgen receptor.

Introduction

Androgens, typically testosterone, are endogenous ligands for the nuclear androgen receptor (AR) and are essential for the development and maintenance of the male reproductive system and secondary male sex characteristics.[1] Androgens play diverse physiological and pathophysiological roles in both males and females.[1,2] Among the pathophysiological effects elicited by androgens, a role as endogenous tumor promoters, especially for prostate tumor, is well known. The effect is considered to be mediated by androgen-binding to AR. The AR is a member of the steroid/thyroid/retinoid/vitamin D_3 nuclear receptor superfamily and acts as an androgen-dependent specific transcription factor.[3,4] The nuclear receptor consists of three main functional domains including the ligand-binding domain (LBD or E-region), DNA-binding domain (DBD or C-region) and amino-terminal domain (A/B region).[4–6] The general structure of the LBD has been elucidated to be composed of 12 α helices and a small β sheet by X-ray crystallography of a number of different nuclear receptors. Among the substructures, the helix H12 plays a critical role in the ligand-dependent activation of the receptor, that is, H12 functions as a lid covering the ligand binding pocket, being in the closed conformation when an agonist occupies the binding pocket and in the open conformation without the agonist or when an antagonist occupies the pocket.[7,8] Recently, the crystal structure of LBD of human AR complexed with an agonist, which coincides with the general structure of LBD (vide supra), was reported.[9]

Androgen antagonists are compounds which antagonize the biological responses induced by endogenous or exogenous androgens, by inhibiting competitively their binding to AR. Because of the known action of androgens as tumor promoters, androgen antagonists are expected to be effective for treatment of these androgen-dependent tumors, especially for prostate tumor.[2] There are two structural types of androgen antagonists which have been used in therapeutics, that is, steroidal and anilide types (Fig. 1). A typical steroidal androgen antagonist is cyproterone acetate (CPA: **1**), which is the first drug to have been used orally, but CPA also interacts with progestin and glucocorticoid receptors.[10,11] Anilide analogues were the first group of non-steroidal androgen antagonists to be discovered, and three of them have been used therapeutically for the treatment of prostate tumor so far.[2,12] Flutamide (**2**) was the first non-steroidal androgen antagonist to enter clinical use, but its active metabolite, 2-hydroxyflutamide (**3**) is cleared so rapidly that it requires frequent dosing (three times a day).[2,13] A cyclic derivative of **2**, nilutamide (**4**),

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can be administered once a day, but it elicits side effects including hot flushes, transient night blindness and alcohol intolerance.[2,14] Bicalutamide (**5**), which is marketed as a recemic mixture with the *R*-isomer being the eutomer, is superior to both flutamide (**2**) and nilutamide (**4**) in terms of its pharmacokinetics and side effect profile.[2,15] Discovery of these non-steroidal androgen antagonists stimulated researchers to develop other structural types of androgen antagonists.

Recently, we reported phthalimide-type androgen antagonists, including compounds **6**–**10**, (Fig. 2).[16–19] These androgen antagonists were derived from DIMP (**11**)[20] and thalidomide, the former of which is a well known androgen antagonist, though not used clinically.[16–19] An anilide analogue **12**, which was derived from tetrafluorophthalimide analogues **6**, **7**, **9** and **10**, also showed anti-androgenic activity.[19] We also reported azo/azoxy-benzene types of androgen antagonists, including compounds **13** and **14**.[19,20] Hamann et al. reported linear tricyclic quinolines, with the generic structure **15** as a novel androgen antagonist scaffold.[2,21,22] In this article, we describe the design, synthesis, and structure–activity relationship study of another structurally novel type of androgen antagonists, 3-substituted (*Z*)-4-(4-*N*,*N*-dialkylaminophenylmethylene)-5(4*H*)-isoxazolones.

Results and Discussion

Molecular design

Androgen antagonists which we wish to develop are ligands which bind the LBD of AR in a competitive manner with androgens. The X-ray crystallographic structure of the LBD of AR,[9] as well as our previous studies on the structural development of retinoids whose target molecule is also a member of the nuclear receptor superfamily,[5,6] suggested that compounds with two aromatic groups connected with two to four carbon unit mimics as a spacer are candidate ligands for AR.[19] Roughly speaking, our androgen antagonists **6**–**9** and **12**–**14**, and a classical androgen antagonist, DIMP (**11**), fall into this structural category. Because all members of the nuclear receptor superfamily are assumed to have

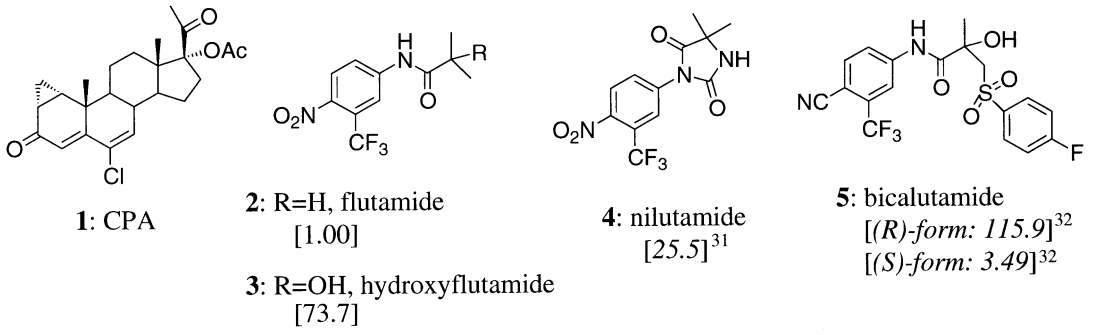


Figure 1. Structures of androgen antagonists used in therapeutics (**1**–**5**). [Values in parentheses are the RBA values (see text). Values in italics are reported by other researchers.]

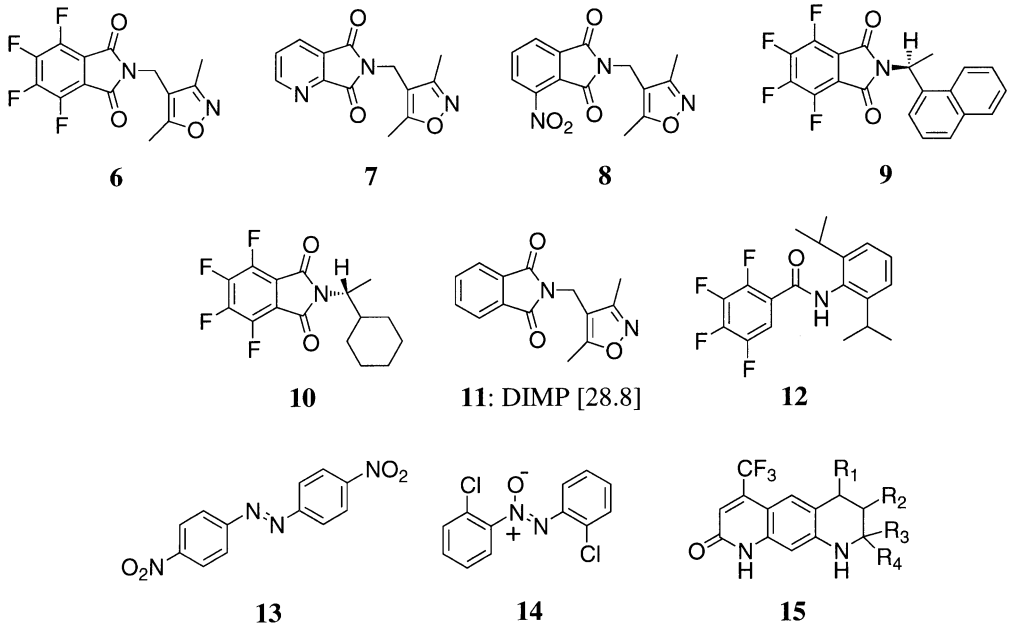


Figure 2. Structures of non-steroidal and non-anilide types of androgen antagonists (**6**–**15**). [A value in parentheses is the RBA value (see text).]

evolved from a single gene and to possess a similarly shaped ligand-binding pocket,[7,23] it might be expected that a ligand superfamily also exists.[8] From such a standpoint, ligands for nuclear receptors should be roughly structurally superimposable, and there should exist a general antagonistic substituent group for nuclear receptor ligands.[8,19] Such a general antagonistic group, if it exists, is expected to hinder conformational change (folding of the helix H12) of the receptor to the active form without reducing the binding affinity of the ligand to which it is attached. Our previous studies on the development of antagonists of nuclear receptor ligands suggested that a dialkylamino or a dialkylaminophenyl group might be such a general antagonistic group, as found in the well known estrogen antagonist, tamoxifen, and other steroid antagonists.[8,19] On this basis, we designed a generic structure **16** as a possible androgen antagonist skeleton (Fig. 3). The structure **16** was constructed through the following steps: (i) extraction of an oxazine substructure from DIMP (**11**), (ii) usage of the oxazine substructure as a spacer linking two aromatic groups, (iii) modification of the oxazine moiety to a more rigid isoxazolone or a pyrazolone substructure, and (iv) introduction of a dialkylamino group as a possible antagonistic group.

Data supporting the idea that a structure related to **16** can be a ligand for AR were obtained by computer-assisted molecular design. A number of compounds were identified as possible ligands for AR by computer-assisted docking search of a database of commercially available compounds (unpublished results). Among the candidate compounds presented by the computer, there was (*Z*)-4-(4-*N,N*-diethylaminophenylmethylene)-3-methyl-5(4*H*)-isoxazolone (**17**). Assays for androgenic and anti-androgenic activity of **17** indicated that **17** is an androgen antagonist with moderate activity (vide infra). Therefore, we sought to develop potent androgen antagonists based on the structures of **16** and **17**.

Synthesis

Compounds **18**–**50** were prepared by the method shown in Figure 4. The isoxazolones (**18**–**23**, **25** and **30**–**50**) and the pyrazolones (**26**–**29**) were synthesized by three-component condensation reaction with benzaldehyde (prepared by Vilsmeier reaction)[24] or acetophenone derivatives, β-keto esters, and hydroxylamine or hydrazines in the presence of phosphoric acid according to the procedures reported by Donleavy and Gilbert.[25] The isoxazolone **24** was synthesized with hydroxylamine and β-keto ester, which was prepared from the condensation reaction of *N*-phenylpyrrolidine and ethyl acetoacetate with formaldehyde. All the compounds were characterized by ^{1}H NMR, mass spectroscopy and elemental analysis or high-resolution mass spectroscopy. The stereochemistries of olefins were determined by means of NOESY experiments (NOE was observed between the methyl at C3 and the vinyl proton at C5 positions).

Biological assay

Androgenic and anti-androgenic activities of the compounds were assessed by using a combination of two assay systems, that is receptor-binding assay and growth promotion/inhibition assay using androgen-dependent Shionogi carcinoma cells SC-3.[15,26,27] The ability of the compounds to be ligands for AR can be assessed by the former assay. The latter assay is to determine whether the compounds are androgen agonists or antagonists. None of the compounds prepared showed growth-promoting activity toward SC-3 cells, suggesting that none was an androgen agonist. Therefore, only growth-inhibitory activities of the compounds (IC_{50} values) toward testosterone-promoted growth of SC-3 cells are presented in this paper (Table 1). Briefly, SC-3 cells were incubated under usual conditions with 10 nM testosterone in the presence of various concentrations of a test compound. The increase of cell number in the absence of test compound was defined as 100%, and relative/expedient anti-androgenic activity of each test compound was presented as IC_{50} value, that is, the concentration at which the testosterone-promoted increase of cell number was reduced to 50%.

For receptor-binding assay, we adopted competitive binding assay using [^{3}H]testosterone and recombinant human AR. The recombinant AR was prepared from cytosol of *Escherichia coli* transformed with a human AR LBD expression vector (GST-hARLBD) which

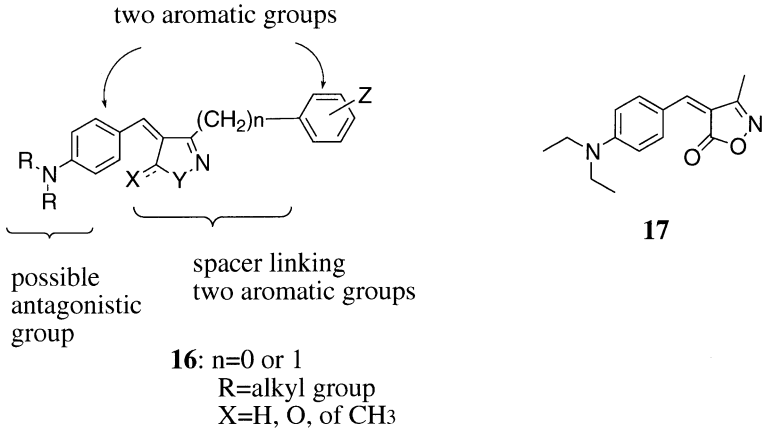


Figure 3. Molecular design of generic structure of androgen antagonists (**16**) and structure of a candidate compound presented by the computer (**17**).

codes amino acids 627–919 of hAR fused with GST protein under the *lac* promoter (see Experimental).[19,28,29] The binding activity of the GST-hARLBD thus prepared toward [^{3}H]testosterone, that is, the K_d (dissociation constant) value, was determined to be 1.4 nM by Scatchard analysis, and this value is close to that of AR prepared from intact SC-3 cells. The binding activity of a test compound was determined by incubation of GST-hARLBD with [^{3}H]testosterone in the presence of various concentrations of the test compound.[30–32] The concentration of a test compound that inhibits [^{3}H]testosterone-binding by 50% (IC_{50} value) was determined after log-logit transformation, and the K_i value was calculated based on the K_d of [^{3}H]testosterone. The K_i value of flutamide (**2**) thus obtained was 1.3 μM. The receptor-binding activity of each test compound is presented as relative binding affinity (RBA), which was defined as (K_i value of flutamide)/(K_i value of test compound) (Figs 5–8).

Table 1. Growth-inhibitory activities toward SC-3 and LNCaP cells

Compd	IC_{50} value (μM)	
	SC-3 cells	LNCaP cells
2: flutamide	2.7	>10[a]
3: hydroxyflutamide	0.8	>10[a]
17	0.6	9.7
19	3.0	>10 (47%)[b]
20	1.6	9.0
21	1.3	>10 (32%)[b]
22	0.4	>10 (38%)[b]
23	3.5	>10 (24%)[b]
26	5.8	9.5
28	1.9	n.t.
29	6.2	>10 (7%)[b]
30	1.6	n.t.
34	2.9	>10 (34%)
35	1.4	>(37%)[b]
36	1.0	5.6
37	0.7	>10 (28%)[b]
38	1.1	>10 (15%)[b]
39	0.3	7.9
40	0.2	>10 (18%)[b]
41	0.4	>10 (38%)[b]
42	0.3	9.8
43	0.4	>10 (22%)[b]
44	0.3	>10 (28%)[b]
45	0.5	>10 (2%)[b]
46	2.9	>10 (24%)[b]
47	6.1	2.5
48	4.0	2.2
49	1.7	1.9
50	3.8	2.1

[a]Known to act as an androgen agonist toward LNCaP cells.
[b]Percent inhibition of testosterone (10 nM)-induced increase of cell growth at 10 μM. n.t., not tested.

For compounds which showed potent anti-androgenic activity, growth inhibition assay using human prostate tumor cells LNCaP was also performed. LNCaP cells are known to possess an aberrant AR with a mutation of 877Thr to Ala, and some androgen antagonists including flutamide (**2**) show androgen-agonistic activity toward LNCaP cells.[29] The data are presented as IC_{50} values measured by the same method used in the case of SC-3 assay (vide supra) (Table 1).

The values (IC_{50} values in SC-3 and LNCaP assays, and RBA values in receptor-binding assay) thus measured showed some deviation from experiment to experiment. However, the results were basically reproducible. Each experiment was performed in triplicate and repeated at least three times, and typical sets of data (mean value of the triplicate) are presented in this paper.

Structure–activity relationship studies (1): (*Z*)-4-(4-dialkylaminophenylmethylene)-3-methyl-5(4*H*)-isoxazolones and their related compounds (17–33) (Fig. 5). As already mentioned, computer-assisted docking search of a commercially available compound database yielded **17** as a candidate ligand. Biological assays indicated that **17** is an androgen antagonist with moderate activity. Therefore, we started our study by modification of the structure of **17**. Structural development was mainly based on receptor (AR)-binding activity, because this reflects direct (intrinsic) activity of the compound, excluding

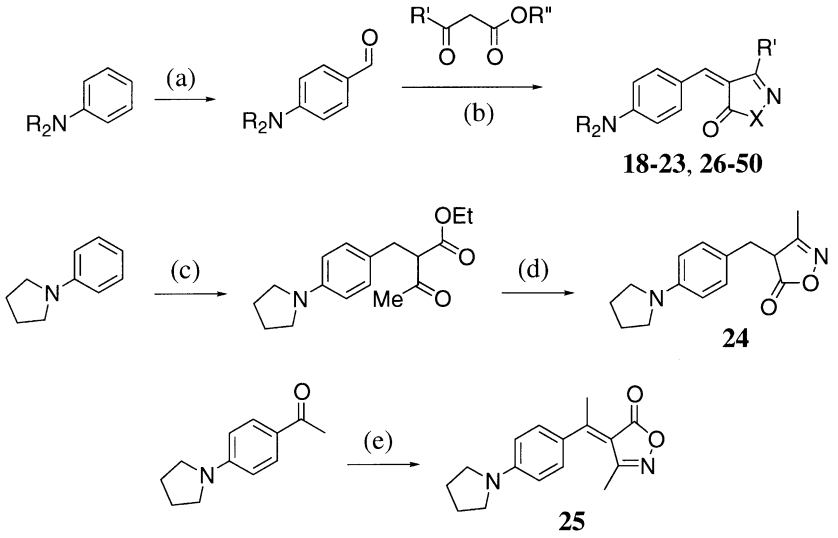


Figure 4. Synthetic methods for isoxazolones (**18**–**25**, and **30**–**50**) and pyrazolones (**26**–**29**).

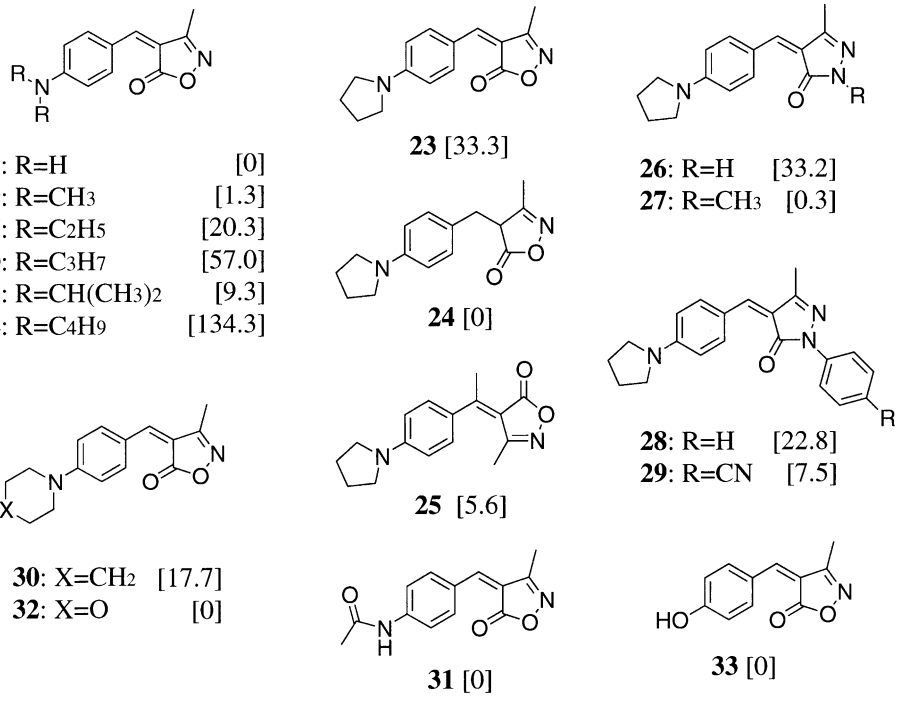


Figure 5. Structures of (*Z*)-4-(*4-N,N*-dialkylaminophenylmethylene)-3-methyl-5(4*H*)-isoxazolones and related compounds (**17**–**33**). [Values in parentheses are the RBA values (*see text*).]

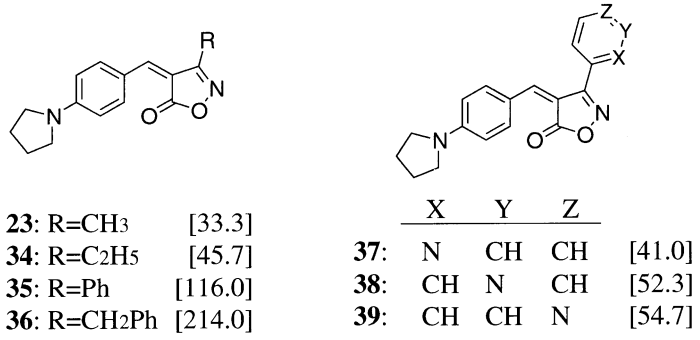


Figure 6. Structures of 3-substituted (*Z*)-4-(4-pyrrolidinophenylmethylene)-5(4*H*)-isoxazolones and related compounds (**34**–**39**). [Values in parentheses are the RBA values (see text).]

involvement of cell permeability, metabolic inactivation, and so on. (Of course, cell level assay is mandatory to determine whether the ligand acts as an agonist or an antagonist.) Therefore, structure–activity relationships are discussed on the basis of RBA values in the following four sections.

The non-alkylated derivative **18**, was inactive. The corresponding hydroxyl analogue **33**, was also inactive. Introduction of two alkyl groups into the amino group (**19**–**23**) resulted in appearance of the activity. The activity of normal alkyl chain-introduced compounds increased in the following order: H: **18** (RBA = 0) $<CH_3$: **19** (1.3) $<C_2H_5$: **17** (20.3) $<C_3H_7$: **20** (57.0) $<C_4H_9$: **22** (134.3). This order is not merely based on the increase of hydrophobicity alone, because the diisopropyl analogue **21** has the weakest activity among compounds **17**–**22**. Cyclization of the diethyl group of **17** resulted in an increase of the activity, that is, **23** with

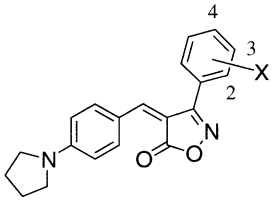


Figure 7. Structures of (*Z*)-4-(4-pyrrolidinophenylmethylene)-3-phenyl-5(4*H*)-isoxazolones and related compounds (**40**–**45**). [Values in parentheses are the RBA values (see text).]

an RBA value of 33.3. However, the ring expansion of **23** to give a piperidino analogue **30** resulted in a decrease of the activity, and introduction of an oxygen atom into the piperidino moiety to give the morpholino analogue **32** abolished the activity. The acetamido analogue **31** corresponding to **18** was also completely inactive.

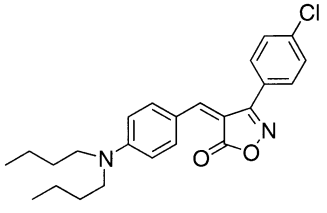
Further structural modification was performed based on **23**, because we anticipated that the very potent activity of **22**, 134.4 times more potent than flutamide (**2**: RBA = 1.00; Fig. 1), might be attributed to its high lipophilicity, not its molecular shape, at least in part. Saturation of the 4-methylene moiety to give **24** resulted in complete disappearance of the activity. The (*E*)-isomer of 3-methylated **23**, that is, **25**, showed moderate activity which was far weaker than that of **23**. An aza analogue **26** corresponding to **23** showed almost the same activity as **23**. Introduction of a substituent onto the nitrogen atom of **26** resulted in a decrease of the activity.

Structure–activity relationship studies (2): 3-substituted (*Z*)-4-(4-pyrrolidinophenylmethylene)-5(4*H*)-isoxazolones and related compounds (34–39) (Fig. 6). Next, based on the ligand superfamily concept (vide supra, Fig. 3), we introduced a second aromatic group into the 3-position of **23**. As expected, introduction of a phenyl group and a benzyl group to give **35** and **36**, respectively, resulted in dramatic increases of the activity, with RBA values of 116.0 and 214.0, respectively. These compounds (**35** and **36**) are 1.6–2.9 times more potent than hydroxyflutamide (**3**: RBA = 73.7; Fig. 1), an active metabolite of flutamide (**2**). They appear to be more active than the (*R*)-form of bicalutamide [eutomer of bicalutamide (**5**: reported RBA = 115.9; Fig. 1)], based on the reported K_i values.[33] Substitution of the 3-methyl group of **23** with an ethyl group to give **34** slightly increased the activity.

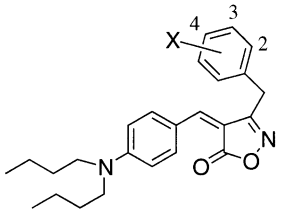
A pyridyl group is also effective as the 3-substituent (**37**–**39**), but less so than a phenyl or a benzyl group. However, pyridyl derivatives are expected to possess higher polarity and hydrophilicity, and might possess superior characteristics as lead compounds for androgen-antagonistic therapeutics (vide infra).

Structure–activity relationship studies (3): effect of a substituent introduced at the 3-phenyl group (40–45) (Fig. 7). The effect of a substituent introduced into the 3-phenyl group of **35** was investigated. Introduction of a halogen atom (fluorine or chlorine) dramatically increased the activity (**40**–**43**). The introduction of chlorine at the *para*-position of **35** to give **41** increased the activity almost 2-fold. Compound **41** showed the most potent activity among the prepared compounds. The *para*-position seems to be a superior position for substitution compared to the *ortho*-position in the case of chloro derivatives; the 4′-chloro derivative **41** (RBA = 220.0) is more potent than the 2′-chloro derivative **40** (158.0). However, the order was reversed in the case of fluorine derivatives; the 2′-fluoro derivative **42** (140.6) is slightly more active than the corresponding 4′-fluoro derivative **43** (125.0). Introduction of another electron-withdrawing group, a nitro group, into **35** to give **44** and **45** moderately decreased the activity. The decreasing effect was more apparent when a nitro group was introduced at the *para*-position (**45**) compared to introduction at the *meta*-position.

Structure–activity relationship studies (4): (*Z*)-4-(4-dibutylaminophenylmethylene)-3-benzyl-5(4*H*)-isoxazolone analogues (46–50) (Fig. 8). The features of the structure–activity relationship of (*Z*)-4-(4-*N,N*-dialkylaminophenylmethylene)-5(4*H*)-isoxazolone derivatives established so far can be summarized as follows. Firstly, in the series of (*Z*)-4-(4-*N,N*-dialkylaminophenylmethylene)-3-methyl-5(4*H*)-isoxazolones (**17**–**23**), the *N,N*-dibutylamino derivative (**22**) shows the most potent receptor-binding affinity (Fig. 5). Secondly, introduction of a phenyl or a benzyl group at the 3-position contributes to potent receptor affinity (Fig. 6). In the series of (*Z*)-4-(pyrrolidinophenylmethylene)-3-phenyl-5(4*H*)-isoxazolones (**35**, **40**–**45**), the 4′-chlorophenyl analogue (**41**) showed the most potent receptor affinity (Fig. 7). Overall these results led us to design hybrid molecules possessing a *N,N*-dibutylaminophenyl group and a 4′-chlorophenyl (**46**) or a chlorinated benzyl group (**48**–**50**). However, these compounds did not show as potent activity as expected. Although a benzyl analogue **47** showed rather potent activity with a RBA value of



46 [67.1]



47: X=H [113.3]
48: X=2-Cl [31.0]
49: X=3-Cl [21.7]
50: X=4-Cl [27.7]

Figure 8. Structures of (*Z*)-4-(4-*N,N*-dibutylaminophenlymethylene)-3-phenyl-5(4*H*)-isoxazolone analogues (**40**–**50**). [Values in parentheses are the RBA values (see text).]

113.2, it was less potent than the corresponding pyrrolidino analogue **36**. Introduction of chlorine (**48**–**50**) dramatically lowered the activity. Surprisingly, the 4′-chlorophenyl analogue (**46**) showed only moderate activity, which is much weaker than those of the corresponding *N*-pyrrolidino analogue **41** and its non-chlorinated analogue **35.** Though we cannot interpret the findings at this stage, one possible explanation might be different conformations of the ligand–receptor complex between the *N,N*-dibutylaminophenyl series (**22**, **46**–**50**) and pyrrolidinophenyl series (**23**–**29, 34**–**45**).

Cell level activity

None of our compounds (**17**–**50**) showed growth-promoting activity toward SC-3 or LNCaP cells cultured in the absence of testosterone, indicating that our compounds do not possess androgen-agonistic activity (data not shown). In addition, under the experimental conditions used, our compounds did not show apparent cytotoxicity. On the other hand, some of our compounds inhibited testosterone-promoted cell growth of SC-3 to the baseline (the cell number was almost the same as that when the cells were incubated in the absence of testosterone), indicating these compounds with AR-affinity are androgen antagonists. The results of growth inhibition assay of testosterone-induced cell growth of SC-3 and LNCaP cells are shown in Table 1.

Concerning SC-3 assay, there seems to be only a rough correlation between the growth-inhibitory activity and AR-affinity (RBA values). However, compounds with potent AR-affinity generally showed potent growth-inhibiting activity. For example, compounds with RBA values more than 120, that is, **22**, **36**, and **40**–**43**, showed potent growth-inhibitory activity on testosterone-induced SC-3 growth with IC_{50} values of less than 1.0 μM, being more potent than flutamide (**2**: $IC_{50} = 2.7$ μM). The IC_{50} values of **22** and **40**–**43** are 0.2–0.4 μM, indicating they are more potent than hydroxyflutamide (**3**: $IC_{50} = 0.8$ μM), a metabolically activated form of flutamide (**2**). All of the potent SC-3 cell growth inhibitors with IC_{50} values of less than 1.0 μM possess RBA values of more than 20. In this sense, **47** showed much weaker SC-3 growth-inhibitory activity ($IC_{50} = 6.1$ μM) than expected from its rather high RBA value (113.3). This discrepancy might be attributed to the highly lipophilic nature of the compound.

Some of our typical compounds were also assessed by LNCaP assay. Generally, LNCaP cells, cloned from human prostate tumor, are resistant to known androgen antagonists, possibly because of their mutated aberrant AR.[33] Flutamide (**2**) is reported to act as an androgen agonist on LNCaP cells. As shown in Table 1, LNCaP cells were rather resistant to our compounds, though some of them (**36**, **39**, and **47**–**50**) acted as androgen antagonists on the cells with IC_{50} values of less than 8 μM. No androgen-agonistic activity was found, as far as checked. The *N,N*-dibutylaminophenyl methylene-3-benzylisoxazolone analogues **47**–**50** showed potent inhibitory activity on testosterone-promoted growth of LNCaP cells with IC_{50} values of 1.9–2.5 μM. The potency of growth-inhibiting activity toward LNCaP cells does not seem to correlate to that toward SC-3 cells. Though we cannot analyze this phenomenon further at this stage, it might be attributed to the difference between AR expressed in SC-3 cells (normal form) and AR expressed in LNCaP cells (mutated aberrant form).[29] These results suggest that our compounds might be superior lead compounds for development of therapeutically useful androgen antagonists with different features from those of flutamide (**2**).

Conclusion

We designed novel non-steroidal/non-anilide type of androgen antagonists based on the ligand superfamily concept and a computer-assisted docking search of a compound database. The molecular design and the structural development studies reached potent androgen antagonists with a (*Z*)-4-(4-pyrrolidinophenylmethylene)-3-phenyl/benzyl-5(4*H*)-isoxazolone structure. Among them, **36** and **41** possess very potent AR-binding affinity, more than 200 times greater than that of flutamide (**2**). They showed potent SC-3 cell growth-inhibitory activity, and they also acted as androgen antagonists toward generally androgen antagonist-resistant LNCaP cells. In the cell level assays, **22**, **37**, and **39**–**45** showed potent SC-3 cell growth-inhibitory activity. Among them, **40**–**43** were revealed to act as androgen antagonists toward LNCaP cells. These compounds are expected to be useful as lead compounds for the development of therapeutically useful androgen antagonists with different types of structures from known androgen antagonists. Further biological evaluation of these compounds is in progress.

Experimental

Chemicals

[1,2,6,7-^{3}H]Testosterone ([^{3}H]testosterone, 101 Ci/mmol), isopropyl β-D-thiogalactoside and Sephadex G-25 were purchased from Amersham Pharmacia Biotech (England). Tris–base, EDTA, glycerol, DTT, sodium chloride, sodium molybdate and testosterone were purchased from Wako Pure Chemical, Ltd. (Japan). Bacto® Trypton and Bacto® Yeast Extract were purchased from Difco Laboratories (USA). Coomassie Protein Assay Kit was purchased from Pierce (USA). Antibody against hAR-LBD was purchased from Santa Cruz Biotechnology, Inc. (USA). Atomlight scintillation cocktail was purchased from Packard Bioscience (Netherlands). MEM (modified Eagle's medium) and flutamide (**2**) were purchased from Sigma-Aldrich Japan K.K. (Japan). FBS (fetal bovine serum) was purchased from Gibco (USA). Cell Counting Kit was purchased from Dojindo (Japan). All other chemicals were purchased from Tokyo Kasei Kogyo Co., Ltd. (Japan), Kanto Chemical Co., Inc. (Japan), Sigma-Aldrich Japan K.K. (Japan) or Lancaster Synthesis (UK).

General synthetic methods. Commercially available reagents were used as supplied. Routine thin layer chromatography (TLC) was performed on silica gel 60 F_{254} plates (Merck, Germany). Column chromatography was performed using silica gel 60 spherical (Kanto Chemical Co., Inc., Japan). Melting points were determined on a MP-J3 melting point apparatus (Yanaco, Japan) and are uncorrected. Fast atom bombardment mass spectra (FAB-MS) were measured by a MS-JEOL JMS-HX110 mass spectrometer using a nitrobenzyl alcohol matrix as appropriate. Proton nuclear magnetic resonance (NMR) spectra data were obtained on a JEOL ALPHA500 spectrometer (500 MHz) and were consistent with assigned structures. Chemical shifts are given in parts per million (ppm) downfield from internal reference TMS in δ units, and coupling constants (*J* values) are given in hertz (Hz). Elemental analysis was performed on a MT-6 elemental analyzer (Yanagimoto, Tokyo).

Preparation of 4 - *N,N* - dipropylaminobenzaldehyde. Under an argon atmosphere, $POCl_3$ (0.770 mL, 8.40 mmol) was slowly added to DMF (5 mL) on an ice bath. To this reaction mixture was added *N,N*-dipropylaniline (1.35 mL, 7.00 mmol), and the resulting mixture was carefully heated at 80 °C for 2 h. The reaction mixture was poured onto cracked ice, neutralized with aqueous sodium hydroxide and extracted with ethyl acetate. The extracts were washed with brine, dried over magnesium sulfate, filtered and concentrated in vacuo. The residue was purified by column chromatography (hexane/ethyl acetate, 10:1) to give 4-*N,N*-dipropylaminobenzaldehyde (1.06 g, 74%) as a white solid.

(*Z*)-4-(4-Aminophenylmethylene)-3-methyl-5(4*H*)-isoxazolone (18). Yield: 23%; orange crystals (CH_2Cl_2); mp 210–211 °C; MS(FAB) *m/z* 203 (MH^+); ^1H NMR (500 MHz, DMSO-d_6) δ 8.35 (d, *J* = 8.5 Hz, 2H), 7.54 (s, 1H), 7.09 (s, 2H), 6.66 (d, *J* = 8.5 Hz, 2H), 2.19 (s, 3H). Anal. calcd for $C_{11}H_{10}N_2O_2$: C, 65.34; H, 4.98; N, 13.85. Found: C, 65.37; H, 5.16; N, 13.67.

(*Z*)-4-(4-*N,N*-Dimethylaminophenylmethylene)-3-methyl-5(4*H*)-isoxazolone (19). Yield: 47%; red needles (EtOAc); mp 207–208 °C; MS(FAB) *m/z* 231 (MH^+), 230 (M^+); ^1H NMR (500 MHz, $CDCl_3$) δ 8.41 (d, *J* = 10.0 Hz, 2H), 7.23 (s, 1H), 6.73 (d, *J* = 10.0 Hz, 2H), 3.16 (s, 6H), 2.25 (s, 3H). Anal. calcd for $C_{15}H_{18}N_2O_2$: C, 67.81; H, 6.13; N, 12.17. Found: C, 67.91; H, 6.40; N, 11.92.

(*Z*)-4-(4-*N,N*-Dipropylaminophenylmethylene)-3-methyl-5(4*H*)-isoxazolone (20). Yield: 42%; red crystals (hexane/EtOAc); mp 117 °C; MS(FAB) *m/z* 287 (MH^+); ^1H NMR (500 MHz, $CDCl_3$) δ 8.4 (brs, 2H), 7.19 (s, 1H), 6.69 (d, *J* = 9.0 Hz, 2H), 3.38 (t, *J* = 8.0 Hz, 4H), 2.25 (s, 3H), 1.68 (tq, *J* = 8.0, 8.0 Hz, 4H), 0.98 (t, *J* = 8.0 Hz, 6H). Anal. calcd for $C_{17}H_{22}N_2O_2$: C, 71.30; H, 7.74; N, 9.78. Found: C, 71.32; H, 7.97; N, 9.65.

(*Z*) - 4 - (4 - *N,N* - Diisopropylaminophenylmethylene) - 3-methyl-5(4*H*)-isoxazolone (21). Yield: 11%; red crystals (hexane/EtOAc); mp 134–135 °C; MS(FAB) *m/z* 287 (MH^+); ^1H NMR (500 MHz, $CDCl_3$) δ 8.36 (d, *J* = 9.0 Hz, 2H), 7.19 (s, 1H), 6.87 (d, *J* = 9.0 Hz, 2H), 4.08 (qq, *J* = 7.0, 7.0 Hz, 2H), 2.25 (s, 3H), 1.38 (d, *J* = 7.0 Hz, 12H). Anal. calcd for $C_{17}H_{22}N_2O_2$: C, 71.30; H, 7.74; N, 9.78. Found: C, 71.28; H, 7.83; N, 9.62.

(*Z*)-4-(4-*N,N*-Dibutylaminophenylmethylene) - 3 - methyl-5(4*H*)-isoxazolone (22). Yield: 75%; red oil; MS(FAB) *m/z* 315 (MH^+); ^1H NMR (500 MHz, $CDCl_3$) δ 8.4 (brs, 2H), 7.14 (s, 1H), 6.68 (d, *J* = 9.5 Hz, 2H), 3.40 (t, *J* = 8.0 Hz, 4H), 2.24 (s, 3H), 1.63 (tt, *J* = 8.0, 8.0 Hz, 4H), 1.39 (tq, *J* = 8.0, 8.0 Hz, 4H), 0.98 (t, *J* = 8.0 Hz, 6H). Anal. calcd for $C_{19}H_{26}N_2O_2$: C, 72.58; H, 8.33; N, 8.91. Found: C, 72.39; H, 8.22; N, 9.05.

(*Z*)-4-(4-pyrrolidinophenylmethylene) - 3 - methyl - 5(4*H*)-isoxazolone (23). Yield: 69%; red needles (benzene); mp 192 °C; MS(FAB) *m/z* 257 (MH^+), 256 (M^+) ;^1H NMR (500 MHz, $CDCl_3$) δ 8.42 (d, *J* = 9.0 Hz, 2H); 7.21 (s, 1H), 6.61 (d, *J* = 9.0 Hz, 2H), 3.47 (tt, *J* = 3.5, 3.5 Hz, 4H), 2.25 (s, 3H), 2.08 (tt, *J* = 3.5, 3.5 Hz, 4H). Anal. calcd for $C_{15}H_{16}N_2O_2$: C, 70.29; H, 6.29; N, 10.93. Found: C, 70.39; H, 6.36; N, 10.86.

Ethyl-2-(4-pyrrolidinophenylmethyl)acetoacetate. A mixture of *N*-phenylpyrrolidine (1.03 g, 7.00 mmol), ethyl acetoacetate (1.82 g, 14.0 mmol) and formalin (3.00 mL of 35%) in acetic acid (7 mL) was stirred at room temperature for 10–12 h. The reaction mixture was neutralized with ice-cold saturated Na_2CO_3 solution and extracted with ethyl acetate. The extracts were washed with brine, dried over magnesium sulfate, filtered and concentrated in vacuo. The residue was purified by column chromatography (hexane/ethyl acetate, 10:1) to give ethyl 2-(4-pyrrolidinophenylmethyl)-acetoacetate (230 mg, 11%). MS(FAB) *m/z* 179 (MH^+), 178 (M^+); ^1H NMR (500 MHz, $CDCl_3$) δ 7.02 (dt, *J* = 8.5, 2.0 Hz, 2H), 6.47 (dt, *J* = 8.5, 2.0 Hz, 2H), 4.16 (q, *J* = 7.0 Hz, 2H), 3.73 (t, *J* = 7.5 Hz, 1H), 3.24 (tt, *J* = 3.5, 3.5 Hz, 4H), 3.06 (t, *J* = 7.5 Hz, 2H), 2.17 (s, 3H), 1.98 (tt, *J* = 3.5, 3.5 Hz, 4H), 1.28 (t, *J* = 7.0 Hz, 3H).

4-(4-Pyrrolidinophenylmethyl)-3-methyl-5(4*H*)-isoxazolone (24). A mixture of ethyl 2-(4-pyrrolidinophenylmethyl)acetoacetate (434 mg, 1.50 mmol), hydroxylamine hydrochloride (206 mg, 3.00 mmol) and sodium acetate (369 mg, 4.50 mmol) in ethanol (30 mL) was heated under reflux for 3 h. The reaction mixture was diluted with ethyl acetate, and the organic layer was washed with brine, dried over magnesium sulfate, filtered and concentrated in vacuo. The residue was purified by column chromatography (hexane/ethyl acetate, 2:1) to give 4-(4-pyrrolidinophenylmethyl) - 3 - methyl - 5(4*H*)-isoxazolone (**24**) (245 mg, 63%) as a brown solid, which was recrystallized from hexane/ethyl acetate; mp 139–142 °C; MS(FAB) *m/z* 259 (MH^+), 258 (M^+); ^1H NMR (500 MHz, $CDCl_3$) δ 7.10 (dd, *J* = 8.0, 1.0 Hz, 2H), 6.48 (dd, *J* = 8.0, 1.0 Hz, 2H), 3.51 (t, *J* = 5.5 Hz, 1H), 3.25 (tt, *J* = 3.5, 3.5 Hz, 4H), 3.16 (dd, *J* = 14.5, 5.5 Hz, 1H), 3.09 (dd, *J* = 14.5, 5.5 Hz, 1H), 1.99 (tt, *J* = 3.5, 3.5 Hz, 4H), 1.99 (s, 3H). HRMS (FAB, MH^+) $C_{15}H_{19}N_2O_2$, calcd for 259.1447, found 259.1484.

(*E*)-4-(4-Pyrrolidinophenyl-1-ethylene)-3-methyl-5(4*H*)-isoxazolone (25). Yield: 33%; orange flakes (EtOAc); mp 147–150 °C; MS(FAB) *m*/*z* 271 (MH^+), 270 (M^+); ^1H NMR (500 MHz, $CDCl_3$) δ 7.20 (dt, *J* = 9.0, 2.0 Hz, 2H), 6.57 (dt, *J* = 9.0, 2.0 Hz, 2H), 3.39 (tt, *J* = 3.5, 3.5 Hz, 4H), 2.80 (s, 3H), 2.07 (tt, *J* = 3.5, 3.5 Hz, 4H), 1.82 (s, 3H). Anal. calcd for $C_{15}H_{16}N_2O_2$: C, 71.09; H, 6.71; N, 10.36. Found: C, 71.09; H, 6.78; N, 10.17. Formation of the corresponding (*Z*)-form was not observed (the stereochemistry of the olefin was determined by means of NOESY experiments). NOE was observed between the methyl at C3 and the aromatic protons at 2′- and 6′-positions.

(*Z*)-4-(4-Pyrrolidinophenylmethylene)-3-methyl-5(4*H*)-pyrazolone (26). Yield: 40%; red crystals (MeOH/EtOAc); mp 213–214 °C; MS(FAB) *m*/*z* 256 (MH^+); ^1H NMR (500 MHz, $CDCl_3$) δ 8.53 (d, *J* = 8.5 Hz, 2H), 8.41 (s, 1H), 7.22 (s, 1H), 6.60 (d, *J* = 8.5 Hz, 2H), 3.44 (tt, *J* = 3.5, 3.5 Hz, 4H), 2.13 (s, 3H), 2.07 (tt, *J* = 3.5, 3.5 Hz, 4H). Anal. calcd for $C_{15}H_{17}N_3O$: C, 70.56; H, 6.71; N, 16.46. Found: C, 70.28; H, 6.86; N, 16.23.

(*Z*)-4-(4-Pyrrolidinophenylmethylene)-1,3-dimethyl-5(4*H*)-pyrazolone (27). Yield: 61%; red crystals (EtOAc); mp 170–172 °C; MS(FAB) *m*/*z* 270 (MH^+), 269 (M^+); ^1H NMR (500 MHz, $CDCl_3$) δ 8.58 (d, *J* = 8.5 Hz, 2H), 7.19 (s, 1H), 6.50 (d, *J* = 8.5 Hz, 2H), 3.45 (tt, *J* = 3.5, 3.5 Hz, 4H), 3.40 (s, 3H), 2.23 (s, 3H), 2.06 (tt, J_H = 3.5, 3.5 Hz, 4H). Anal. calcd for $C_{16}H_{19}N_3O$: C, 71.35; H, 7.11; N, 15.60. Found: C, 71.36; H, 7.19; N, 15.51.

(*Z*)-4-(4-Pyrrolidinophenylmethylene)-1-phenyl-3-methyl-5(4*H*)-pyrazolone (28). Yield: 47%; red crystals (EtOAc); mp 205 °C; MS(FAB) *m*/*z* 332 (MH^+), 332 (M^+); ^1H NMR (500 MHz, $CDCl_3$) δ 8.59 (d, *J* = 8.5 Hz, 2H), 8.01 (dd, *J* = 8.0, 1.0 Hz, 2H), 7.41 (td, *J* = 8.0, 1.0 Hz, 2H), 7.15 (td, *J* = 8.0, 1.0 Hz, 2H), 6.60 (d, *J* = 8.5 Hz, 2H), 3.45 (tt, *J* = 3.5, 3.5 Hz, 4H), 2.33 (s, 3H), 2.07 (tt, *J* = 3.5, 3.5 Hz, 4H). Anal. calcd for $C_{21}H_{21}N_3O$: C, 76.11; H, 6.39; N, 12.68. Found: C, 76.25; H, 6.55; N, 12.58

(*Z*)-4-(4-Pyrrolidinophenylmethylene)-1-(4-cyanophenyl)-3-methyl-5(4*H*)-pyrazolone (29). Yield: 67%; red needles (CH_2Cl_2/EtOAc); mp 235–236 °C; MS(FAB) *m*/*z* 357 (MH^+), 356 (M^+); ^1H NMR (500 MHz, $CDCl_3$) δ 8.55 (d,*J* = 8.5 Hz, 2H), 8.25 (dt,*J* = 8.0, 2.0 Hz, 2H), 7.67 (dt,*J* = 9.0, 2.0 Hz, 2H), 7.28 (s, 1H), 6.64 (d,*J* = 8.5 Hz, 2H), 3.47 (tt, *J* = 3.5, 3.5 Hz, 4H), 2.09 (s, 3H), 2.07 (tt, *J* = 3.5, 3.5 Hz, 4H). Anal. calcd for $C_{22}H_{20}N_4O$: C, 74.14; H, 5.66; N, 15.72. Found: C, 74.20; H, 5.95; N, 15.75.

(*Z*)-4-(4-Piperazinophenylmethylene)-3-methyl-5(4*H*)-isoxazolone (30). Yield: 34%; orange needles (hexane/EtOAc); mp 163 °C; MS(FAB) *m*/*z* 271 (MH^+); ^1H NMR (500 MHz, $CDCl_3$) δ 8.39 (d, *J* = 9.0 Hz, 2H), 7.20 (s, 1H), 6.87 (d, *J* = 9.0 Hz, 2H), 3.5 (brs, 4H), 1.7 (brs, 6H). Anal. calcd for $C_{16}H_{18}N_2O_2$; C, 71.09; H, 6.71; N, 10.36. Found: C, 70.97; H, 6.88; N, 10.45.

(*Z*)-4-(4-Acetoaminophenylmethylene)-3-methyl-5(4*H*)-isoxazolone (31). Yield: 41%; yellow solid (MeOH/EtOAc); mp 253–257 °C (decomposition); MS(FAB) *m*/*z* 245 (MH^+); ^1H NMR (500 MHz, DMSO-d_6) δ 10.48 (s, 1H), 8.46 (d, *J* = 8.5 Hz, 2H), 7.83 (s, 1H), 7.77 (d, J_H = 8.5 Hz, 2H), 2.26 (s, 3H), 2.11 (s, 3H). Anal. calcd for $C_{13}H_{12}N_2O_2$: C, 63.93; H, 4.95; N, 11.47. Found: C, 63.82; H, 5.06; N, 11.35.

(*Z*)-4-(4-Morpholinophenylmethylene)-3-methyl-5(4*H*)-isoxazolone (32). Yield: 46%; red needles (CH_2Cl_2); mp 202 °C; MS(FAB) *m*/*z* 273 (MH^+); ^1H NMR (500 MHz, $CDCl_3$) δ 8.42 (d, *J* = 9.0 Hz, 2H), 7.25 (s, 1H), 6.90 (d, *J* = 9.0 Hz, 2H), 3.86 (t, *J* = 4.5 Hz, 4H), 3.86 (t, *J* = 4.5 Hz, 4H), 2.26 (s, 3H). Anal. calcd for $C_{15}H_{16}N_2O_3$: C, 66.16; H, 5.92; N, 10.29. Found: C, 65.93; H, 5.98; N, 10.27.

(*Z*)-4-(4-hydroxyphenylmethylene)-3-methyl-5(4*H*)-isoxazolone (33). Yield: 40%; yellow flakes (EtOAc); mp 207–217 °C; MS(FAB) *m*/*z* 204 (MH^+); ^1H NMR (500 MHz, $CDCl_3$) δ 8.45 (d, *J* = 9.0 Hz, 2H), 7.66 (s, 1H), 6.90 (td, *J* = 9.0, 1.5 Hz, 2H), 3.30 (tt, *J* = 1.5, 1.5 Hz, 1H), 2.26 (s, 3H). Anal. calcd for $C_{11}H_9NO_3$: C, 65.02; H, 4.46; N, 6.89. Found: C, 64.95; H, 4.52; N, 6.73.

Synthesis of (*Z*)-4-(4-pyrrolidinophenylmethylene)-3-ethyl-5(4*H*)-isoxazolone (34). Phosphoric acid (2.94 g) was added to a mixture of 4-pyrrolidinobenzaldehyde (526 mg, 3.00 mmol) and 3-keto-*n*-valerate (1.95 g, 15.0 mmol), and the whole mixture was stirred at room temperature for 10–12 h. The reaction mixture was poured into ice-cold sodium hydroxide solution and extracted with dichloromethane. The extracts were washed with brine, dried over magnesium sulfate, filtered and concentrated in vacuo. The residue was purified by column chromatography (hexane/ethyl acetate, 3:2) to give (*Z*)-4-(4-pyrrolidinophenylmethylene)-3-ethyl-5(4*H*)-isoxazolone (**34**) (171 mg, 21%) as red crystals, which were further purified by recrystallization from hexane/ethyl acetate to give orange crystals; mp 147 °C; MS(FAB) *m*/*z* 271 (MH^+), 271 (M^+); ^1H NMR (500 MHz, $CDCl_3$) δ 8.41 (d, *J* = 8.5 Hz, 2H), 7.23 (s, 1H), 6.60 (d, *J* = 8.5 Hz, 2H), 3.47 (tt, *J* = 3.5, 3.5 Hz, 4H), 2.64 (q, *J* = 7.5 Hz, 2H), 2.09 (tt, *J* = 3.5, 3.5 Hz, 4H), 1.34 (t, *J* = 7.5 Hz, 3H). Anal. calcd for $C_{16}H_{18}N_2O_2$: C, 71.09; H, 6.71; N, 10.36. Found: C, 71.00; H, 6.82; N, 10.21.

(*Z*)-4-(4-Pyrrolidinophenylmethylene)-3-phenyl-5(4*H*)-isoxazolone (35). Yield: 81%; red crystals (CH_2Cl_2/EtOAc); mp 172–173 °C; MS(FAB) *m*/*z* 319 (MH^+), 318 (M^+); ^1H NMR (500 MHz, $CDCl_3$) δ 8.4 (brs, 2H), 7.5–7.6 (m, 5H), 7.370 (s, 1H), 6.59 (d, *J* = 9.0 Hz, 2H), 3.47 (tt, *J* = 3.0, 3.0 Hz, 4H), 2.08 (tt, *J* = 3.0, 3.0 Hz, 4H). Anal. calcd for $C_{20}H_{18}N_2O_2$: C, 75.45; H, 5.70; N, 8.80. Found: C, 75.35; H, 5.82; N, 8.69.

(*Z*)-4-(4-Pyrrolidinophenylmethylene)-3-phenylmethyl-5(4*H*)-isoxazolone (36). Yield: 82%; orange flakes (CH_2Cl_2/EtOAc); mp 196–197 °C; MS(FAB) *m*/*z* 333 (MH^+), 332 (M^+); ^1H NMR (500 MHz, $CDCl_3$) δ 8.2

(brs, 2H), 7.3–7.4 (m, 5H), 7.16 (s, 1H), 6.54 (d, $J=8.5$ Hz, 2H), 3.98 (s, 2H), 3.44 (tt, $J=3.0$, 3.0 Hz, 4H), 2.06 (tt, $J_H=3.0$, 3.0 Hz, 4H). Anal. calcd for $C_{21}H_{20}N_2O_2$: C, 75.88; H, 6.06; N, 8.43. Found: C, 75.79; H, 6.11; N, 8.34.

(*Z*)-4-(4-Pyrrolidinophenylmethylene) - 3 - (2 - pyridyl) - 5(4*H*) - isoxazolone (37). Yield: 16%; red crystals (hexane/EtOAc); mp 180–181 °C; MS(FAB) *m/z* 320 (MH^+); ^1H NMR (500 MHz, $CDCl_3$) δ 8.77 (s, 1H), 8.75 (dd, $J=5.0$, 1.5 Hz, 1H), 8.50 (d, $J=9.0$ Hz, 2H), 8.03 (d, $J=8.0$ Hz, 1H), 7.84 (td, $J=8.0$, 1.5 Hz, 1H), 7.40 (ddd, $J=8.0$, 5.0, 1.0 Hz, 1H), 6.61 (d, $J=9.0$ Hz, 2H), 3.48 (tt, $J=3.5$, 3.5 Hz, 4H), 2.09 (tt, $J=3.5$, 3.5 Hz, 4H). Anal. calcd for $C_{19}H_{17}N_3O_2$: C, 71.46; H, 5.37; N, 13.16. Found: C, 71.61; H, 5.58; N, 12.92.

(*Z*)-4-(4-Pyrrolidinophenylmethylene) - 3 - (3 - pyridyl) - 5(4*H*) - isoxazolone (38). Yield: 49%; red needles (CH_2Cl_2/EtOAc); mp 145–146 °C; MS(FAB) *m/z* 320 (MH^+); ^1H NMR (500 MHz, $CDCl_3$) δ 8.85 (d, $J=1.5$ Hz, 1H), 8.79 (dd, $J=5.0$, 1.5 Hz, 1H), 8.4 (brs, 2H), 7.95 (dt, $J=8.0$, 1.5 Hz, 1H), 7.40 (ddd, $J=8.0$, 5.0, 0.5 Hz, 1H), 7.31 (s, 1H), 6.61 (d, $J=9.0$ Hz, 2H), 3.49 (tt, $J=3.5$, 3.5 Hz, 4H), 2.09 (tt, $J=3.5$, 3.5 Hz, 4H). Anal. calcd for $C_{19}H_{17}N_3O_2$: C, 71.46; H, 5.37; N, 13.16. Found: C, 71.53; H, 5.55; N, 12.91.

(*Z*)-4-(4-Pyrrolidinophenylmethylene) - 3 - (4 - pyridyl) - 5(4*H*) - isoxazolone (39). Yield: 51%; red needles (CH_2Cl_2/EtOAc); mp 210–211 °C; MS(FAB) *m/z* 320 (MH^+); ^1H NMR (500 MHz, $CDCl_3$) δ 8.81 (dd, $J_H=6.0$, 0.5 Hz, 2H), 8.4 (brs, 2H), 7.52 (dd, $J=6.0$, 0.5 Hz, 2H), 7.32 (s, 1H), 6.61 (d, $J=9.0$ Hz, 2H), 3.50 (tt, $J=3.5$, 3.5 Hz, 4H), 2.10 (tt, $J=3.5$, 3.5 Hz, 4H). Anal. calcd for $C_{19}H_{17}N_3O_2$: C, 71.46; H, 5.37; N, 13.16. Found: C, 71.26; H, 5.44; N, 13.46.

(*Z*)-4-(4-Pyrrolidinophenylmethylene)-3-(2-chlorophenyl) - 5(4*H*) - isoxazolone (40). Yield: 35%; red crystals (EtOAc); mp 220–221 °C; MS(FAB) *m/z* 353 (MH^+); ^1H NMR (500 MHz, $CDCl_3$) δ 8.4 (brs, 2H), 7.54 (dd, $J=7.5$, 1.0 Hz, 1H), 7.49 (dd, $J=7.5$, 1.0 Hz, 1H), 7.48 (td, $J=7.5$, 1.0 Hz, 1H), 7.42 (td, $J=7.5$, 1.0 Hz, 1H), 6.97 (s, 1H), 6.58 (d,$J=9.0$ Hz, 2H), 3.47 (tt, $J=3.5$, 3.5 Hz, 4H), 2.08 (tt, $J=3.5$, 3.5 Hz, 4H). Anal. calcd for $C_{20}H_{17}N_2O_2Cl$; C, 68.09; H, 4.86; N, 7.94. Found: C, 68.36; H, 5.08; N, 7.90.

(*Z*)-4-(4-Pyrrolidinophenylmethylene)-3-(4-chlorophenyl) - 5(4*H*) - isoxazolone (41). Yield: 62%; red needles (CH_2Cl_2/EtOAc); mp 170–174 °C; MS(FAB) *m/z* 353 (MH^+); ^1H NMR (500 MHz, $CDCl_3$) δ 8.4 (brs, 2H), 7.54 (dd, $J=7.0$, 2.5 Hz, 2H), 7.51 (dd, $J=7.0$, 2.5 Hz, 2H), 7.31 (s, 1H), 6.60 (d, $J=9.0$ Hz, 2H), 3.48 (tt, $J=3.5$, 3.5 Hz, 4H), 2.09 (tt, $J=3.5$, 3.5 Hz, 4H). Anal. calcd for $C_{20}H_{17}N_2O_2Cl$: C, 68.09; H, 4.86; N, 7.94. Found: C, 68.08; H, 5.11; N, 8.06.

(*Z*)-4-(4-Pyrrolidinophenylmethylene)-3-(2-fluorophenyl)-5(4*H*)-isoxazolone (42). Yield: 48%; red needles (hexane/EtOAc); mp 200–201 °C; MS(FAB) *m/z* 337 (MH^+); ^1H NMR (500 MHz, $CDCl_3$) δ 8.4 (brs, 2H), 7.55 (d,$J=7.5$ Hz, 1H), 7.53 (d, $J=9.0$ Hz, 1H), 7.31 (d, $J=7.5$ Hz, 1H), 7.23 (d, $J=9.0$ Hz, 1H), 7.14 (d, $J=2.0$ Hz, 1H), 6.65 (d, $J=9.0$ Hz, 2H), 3.47 (tt, $J=3.5$, 3.5 Hz, 4H). 2.08 (tt, $J=3.5$, 3.5 Hz, 4H). Anal. calcd for $C_{20}H_{17}N_2O_2F$; C, 71.42: H, 5.09; N, 8.33. Found: C, 71.46; H, 5.26; N, 8.12.

(*Z*)-4-(4-Pyrrolidinophenylmethylene)-3-(4-fluorophenyl)-5(4*H*)-isoxazolone (43). Yield: 53%; purple crystals (hexane/EtOAc); mp 217 °C; MS(FAB) *m/z* 337 (MH^+); ^1H NMR (500 MHz, $CDCl_3$) δ 8.4 (brs, 2H), 7.58 (ddd, $J=8.5$, 5.5, 2.0 Hz, 2H), 7.31 (s, 1H), 7.23 (td, $J=8.5$, 2.0 Hz, 2H), 6.60 (d, $J=9.0$ Hz, 2H), 3.48 (tt, $J=3.5$, 3.5 Hz, 4H), 2.09 (tt, $J=3.5$, 3.5 Hz, 4H). Anal. calcd for $C_{20}H_{17}N_2O_2F$; C, 71.42: H, 5.09; N, 8.33. Found: C, 71.51; H, 5.29; N, 8.21.

(*Z*)-4-(4-Pyrrolidinophenylmethylene)-3-(3-nitrophenyl)-5(4*H*) - isoxazolone (44). Yield: 79%; red crystals (CH_2Cl_2/MeOH); mp 215 °C; MS(FAB) *m/z* 364 (MH^+); ^1H NMR (500 MHz, $CDCl_3$) δ 8.47 (t, $J=2.0$ Hz, 1H), 8.41 (ddd, $J=9.0$, 2.0, 1.0 Hz, 2H), 7.96 (dt, $J=8.0$, 1.0 Hz, 1H), 7.74 (d, $J=8.0$ Hz, 1H), 7.29 (s, 1H), 6.61 (d, $J=9.0$ Hz, 2H), 3.50 (tt, $J=3.5$, 3.5 Hz, 4H), 2.10 (tt, $J=3.5$, 3.5 Hz, 4H). Anal. calcd for $C_{20}H_{17}N_3O_4$: C, 66.11; H, 4.92; N, 11.56. Found: C, 66.00; H, 4.92; N, 11.84.

(*Z*)-4-(4-Pyrrolidinophenylmethylene)-3-(4-nitrophenyl)-5(4*H*) - isoxazolone (45). Yield: 73%; red crystals (CH_2Cl_2); mp 178–180 °C; MS(FAB) *m/z* 364 (MH^+); ^1H NMR (500 MHz, DMSO-d_6) δ 8.5 (brs, 2H), 8.40 (dt, $J=9.0$, 1.0 Hz, 2H), 7.92 (dt, $J=9.0$, 1.0 Hz, 2H), 7.48 (s, 1H), 6.74 (d, $J=9.0$ Hz, 2H), 3.49 (tt, $J=3.5$, 3.5 Hz, 4H), 1.99 (tt, $J=3.5$, 3.5 Hz, 4H). Anal. calcd for $C_{20}H_{17}N_3O_4$: C, 66.11; H, 4.92; N, 11.56. Found: C, 65.81; H, 4.90; N, 11.37.

(*Z*)-4-(4-*N*,*N*-Dibutylaminophenylmethylene)-3-(4-chlorophenyl) - 5(4*H*) - isoxazolone (46). Yield: 62%; red oil; MS(FAB) *m/z* 411 (MH^+); ^1H NMR (500 MHz, $CDCl_3$) δ 8.4 (brs, 2H), 7.53 (dd, $J=7.0$, 2.5 Hz, 2H), 7.51 (dd, $J=7.0$, 2.5 Hz, 2H), 7.28 (s, 1H), 6.67 (d, $J=9.0$ Hz, 2H), 3.41 (t, $J=7.5$ Hz, 4H), 1.63 (tt, $J=7.5$ Hz, 4H), 1.39 (tq, $J=7.5$, 7.5 Hz, 4H), 0.98 (t, $J=7.5$ Hz, 6H). HRMS (FAB, MH^+) $C_{24}H_{28}N_2O_2{}^{35}Cl$, calcd for 411.1839, found 411.1849.

(*Z*)-4-(4-*N*,*N*-Dibutylaminophenylmethylene) - 3 - phenylmethyl-5(4*H*)-isoxazolone (47). Yield: 48%; red needles (hexane/EtOAc); mp 118 °C; MS(FAB) *m/z* 391 (MH^+); ^1H NMR (500 MHz, $CDCl_3$) δ 8.25 (brs, 2H), 7.33–7.25 (m, 4H), 7.13 (s, 1H), 6.62 (d,$J=9.4$ Hz, 2H), 3.97 (s, 2H), 3.37 (m, 4H), 1.59 (m, 4H), 1.36 (m, 4H), 0.96 (t, $J=7.4$ Hz, 6H). Anal. calcd for $C_{25}H_{30}N_2O_2$: C, 76.89; H, 7.74; N, 7.17. Found: C, 76.80; H, 7.82; N, 7.17.

(*Z*)-4-(4-*N*,*N*-Dibutylaminophenylmethylene)-3-(2-chlorophenylmethyl) - 5(4*H*)-isoxazolone (48). Yield: 56%; orange solid (hexane/EtOAc); mp 101–117 °C; MS(FAB) *m/z* 425 (MH^+); ^1H NMR (500 MHz, $CDCl_3$) δ 8.31 (brs, 2H), 7.41 (d, $J=7.3$, 6.9 Hz, 2H), 7.36–7.2 (m, 2H), 7.22 (s, 1H), 6.64 (d, $J=9.4$ Hz, 2H),

4.10 (s, 2H), 3.38 (m, 4H), 1.60 (m, 4H), 1.35 (m, 4H), 0.96 (t, $J=7.3$ Hz, 6H). Anal. calcd for $C_{25}H_{29}N_2O_2Cl$: C, 70.66; H, 6.88; N, 6.59. Found; C, 70.49; H, 6.93; N, 6.48.

(*Z*)-4-(4-*N*,*N*-Dibutylaminophenylmethylene)-3-(3-chlorophenylmethyl) - 5(4*H*) - isoxazolone (49). Yield: 52%; orange flakes (hexane/EtOAc); mp 101 °C; MS(FAB) *m*/*z* 425 (MH^+); ^1H NMR (500 MHz, $CDCl_3$) δ 8.27 (brs, 2H), 7.4–7.2 (m, 4H), 7.09 (s, 1H), 6.63 (d, $J=9.4$ Hz, 2H), 3.94 (s, 2H), 3.38 (m, 4H), 1.60 (m, 4H), 1.37 (m, 4H), 0.96 (t, $J=7.2$ Hz, 6H). Anal. calcd for $C_{25}H_{29}N_2O_2Cl$: C, 70.66; H, 6.88; N, 6.59. Found: C, 70.54; H, 6.96; N, 6.54.

(*Z*)-4-(4-*N*,*N*-Dibutylaminophenylmethylene)-3-(4-chlorophenylmethyl) - 5(4*H*) - isoxazolone (50). Yield: 43%; orange flakes (hexane/EtOAc); mp 123–124 °C; MS(FAB) *m*/*z* 425 (MH^+); ^1H NMR (500 MHz, $CDCl_3$) δ 8.26 (brs, 2H), 7.3–7.2 (m, 4H), 7.07 (s, 1H), 6.63 (d, $J=9.4$ Hz, 2H), 3.94 (s, 2H), 3.38 (m, 4H), 1.60 (m, 4H), 1.37 (m, 4H), 0.96 (t, $J=7.2$ Hz, 6H). Anal. calcd for $C_{25}H_{29}N_2O_2Cl$: C, 70.66; H, 6.88; N, 6.59. Found: C, 70.64; H, 6.94; N, 6.57.

Receptor binding assay

Binding affinities of test compounds for hAR (human androgen receptor) were measured in competition experiments using [^{3}H]testosterone and cytosolic fraction of hAR-LBD (hAR ligand-binding domain)-transformed *E. coli* as described previously.[20,28,30,31] A hAR-LBD expression plasmid vector which codes GST-hARLBD (627-919 aa, EF domain) fusion protein under the *lac* promoter (provided by Prof. S. Kato, Univ. of Tokyo) was transfected into *E. coli* strain *HB-101*. An overnight culture (10 mL) of the bacteria was added to 1 L of LB medium and incubated at 27 °C until its optical density reached 0.6–0.7 at 600 nm. Following the addition of IPTG to a concentration of 1 mM, incubation was continued for an additional 4.5 h. Cells were harvested by centrifugation at 4000*g* at 4 °C for 15 min and stored at −80 °C until use. All subsequent operations were performed at 4 °C. The bacterial pellet obtained from 40 mL of culture was resuspended in 1 mL of ice-cold TEGDM buffer (10 mM Tris, 1 mM EDTA, 10% glycerol, 10 mM DTT, 10 mM sodium molybdate). This suspension was subjected to sonication using 12×7 s bursts on ice (USP-600A sonicator, Shimadzu, Japan) and crude GST-hARLBD fraction was prepared by centrifugation of the suspension at 12,000*g* for 30 min at 4 °C. Western blot analysis showed that GST-hARLBD existed in the supernatant cytosolic fraction. This crude receptor fraction was diluted to a protein concentration of 0.3–0.5 mg/mL and used in binding assays as GST-hARLBD fraction. Total protein was determined using a Coomassie Protein Assay Kit. In preliminary experiments, the equilibrium dissociation constant (K_d) of testosterone was determined by incubating the GST-hARLBD fraction with increasing concentrations of [^{3}H]testosterone (from 0.1 to 100 nM final concentration) at 4 °C for 12–18 h. Non-specific binding was assessed by addition of 1000-fold excess of nonradioactive testosterone. Separation of ligand-bound protein fraction and free radioactive ligand was achieved by Sephadex G-25 gel filtration column chromatography. The protein fraction was collected and the radioactivity was determined with a liquid scintillation counter. Under the experimental conditions, 10 nM [^{3}H]testosterone was enough to saturate the binding sites in the GST-hARLBD fraction. All experiments were performed in triplicate or more. To determine the K_d of [^{3}H]testosterone, data were analyzed using a modified form of the Scatchard equation: $B/F=(-1/K_d)\times B+B_{max}/K_d$, where B is the concentration of specifically bound [^{3}H]testosterone, F is the concentration of free [^{3}H]testosterone, and B_{max} is the maximum concentration of available binding sites. Binding competition studies for the test compounds were then performed under identical conditions by incubating increasing concentrations (100 nM to 10 μM) of test compound (dissolved in DMSO) with the GST-hARLBD fraction in the presence of a saturating concentration of [^{3}H]testosterone (10 nM) at 4 °C for 12–18 h. The concentration of test compounds that inhibited [^{3}H]testosterone-binding to the extent of 50% was quantified (IC_{50}) after log–logit transformation. The K_i values were determined by application of the Cheng–Prussof equation to the IC_{50} values, where $K_i=IC_{50}/\{1+([^3H]\text{testosterone})/K_d\}$. Relative binding affinity (RBA) was defined as: $RBA=K_{iFlu}/K_i$, where K_{iFlu} is K_i of flutamide (**2**), the reference compound.

SC-3/LNCaP growth inhibition assay

Shionogi Carcinoma-3 (SC-3) cells were cloned from Shionogi Carcinoma 115 cells, which were established from a mouse breast cancer. SC-3 shows androgen-dependent growth.[26,27] In this assay, androgenic and anti-androgenic activities of test compounds were determined in terms of SC-3 growth promotion and inhibition, respectively. SC-3 cells were cultured in the presence of MEM supplemented with 10% FBS and 10 nM testosterone at 37 °C 5% CO_2. All experiments were performed in triplicate or more. For SC-3 cell growth-inhibition assay, the cells were trypsinized, and diluted to 3.0×10^4 cell/mL with MEM supplemented with 10% charcoal resin-stripped fetal bovine serum. This cell suspension was transferred to 96-well microtiter plates, and various concentrations of test compound (from 100 nM to 10 μM DMSO solution) and/or testosterone ethanol solution (final concentration 10 nM) were added. Then the plates were incubated at 37 °C 5% CO_2 for 3 days, and the cell number was determined using the WST-1 method with a Cell Counting Kit and an MPR-A4i2 micro plate reader (TOSOH, Japan). The number of cells on wells with testosterone alone was defined as 100%. The concentration of test compounds that inhibited the increase of the cell number induced by 10 nM testosterone by 50% was quantified (IC_{50}) after log–logit transformation. LNCaP growth promotion and inhibition assays were performed by the same method as described for SC-3 assay, except that RPMI1640 medium was used.

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