Anti-Androgenic Activity of Substituted Azo- and Azoxy-Benzene Derivatives

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Substituted phenylazo and phenylazoxy compounds were systematically prepared and their anti-androgenic activity was measured in terms of (1) the growth-inhibiting effect on an androgen-dependent cell line, SC-3, and (2) the binding affinity to nuclear androgen receptor. Generally, azo/azoxy compounds showed cell toxicity, and the growth-inhibiting effects on SC-3 cells correlated with the toxicity. However, some compounds, including 4,4′-dinitroazobenzene (25), 4,4′-dimethoxyazobenzene (33), and 2,2′-dichloroazoxybenzene (47), possessed potent anti-androgenic activity without apparent cell toxicity.

Keywords anti-androgen; azobenzene; azoxybenzene; androgen receptor; structure–activity relationship

Androgens, a steroidal sex hormones, are known to act as endogenous tumor promoters, especially for prostate cancer.[1,2] Therefore, anti-androgens which antagonize the biological responses induced by endogenous or exogenous androgen are expected to be effective for the treatment of androgen-dependent tumors. So far, two structural types of anti-androgens, *i.e.*, steroidal and nonsteroidal types, are known (**1**—**5**) (Fig. 1). However, the known anti-androgens have rather poor potency and specificity.[3—5] For example, cryptoteron acetate (**1**), the first steroidal anti-androgen to have been used in therapeutics, also interacts with progestin and glucocorticoid receptors,[6,7] and nonsteroidal anti-androgens, flutamide (**2**), anandron (**3**), casodex (**4**) and DIMP (**5**), bind relatively weakly to nuclear androgen receptor.[6,8] To overcome these problems, we need to find structurally novel anti-androgens.

Recently, we have reported novel and potent nonsteroidal anti-androgens with a phthalimide skeleton, which were structurally derived from thalidomide.[3—5] During the structural development studies, we found that some substituted aromatic amides showed moderate anti-androgenic activity.[4] Because an amide group can be regarded as a geometrical isoster of an azo or an azoxy group,[9] we started to investigate the anti-androgenic activity of substituted aromatic azo/azoxy derivatives (**6**—**66**, Fig. 2, Tables 1 and 2).

MATERIALS AND METHODS

Chemicals Substituted azobenzenes (**7**—**29**, **31**—**36**, Fig. 2 and Table 1) were prepared by oxidation of the corresponding substituted anilines with manganese dioxide. Typically, a substituted aniline (200 mg) and manganese dioxide (2 g) in toluene (20 ml) were refluxed for 1—3 h. The mixture was filtered through Celite and the filtrate was evaporated under reduced pressure. The resultant residue was purified by silica gel column chromatography (and recrystallization) to give the corresponding azobenzene derivative in good yield (70—90%). The structure was confirmed by ^{1}H-NMR, mass spectroscopy and elemental analysis. ^{1}H-NMR spectroscopic study in $CDCl_3$ showed that the azobenzene derivatives thus prepared exist predominantly in *trans*-form with 10 to 20% of *cis*-form under the equilibrium state (Concerning the crystalline derivatives, only *trans*-forms were detected when the ^{1}H-NMR spectroscopic studies were performed immediately after the corresponding crystals were dissolved in $CDCl_3$).

Azoxybenzene derivatives (**39**—**59**, **61**—**66**, Fig. 2 and Table 2) were prepared by oxidation of the corresponding substituted azobenzenes with *m*-chloroperbenzoic acid (mCPBA). Typically, to a solution of substituted azobenzene (1 eq) in dichloroethane was added mCPBA (3 eq). The mixture was refluxed for 3—5 h, washed successively with saturated aqueous $NaHCO_3$, distilled water, and saturated aqueous NaCl, then dried over $MgSO_4$, and evaporated. The residue was purified by silica gel column chromatography (and recrystallization). The structures were confirmed by ^{1}H-NMR, mass spectroscopy and elemental analysis. In the con-

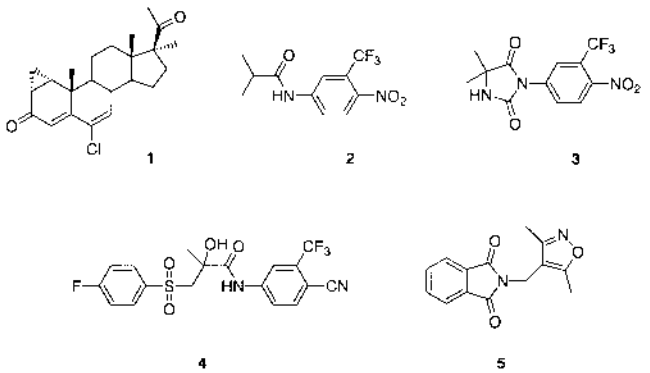

Fig. 1. Structures of Cryptoteron Acetate (**1**), Flutamide (**2**), Anandron (**3**), Casodex (**4**) and DIMP (**5**)

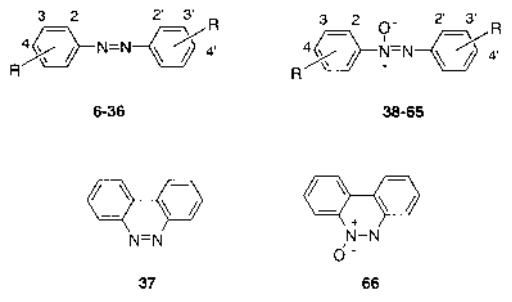

Fig. 2. Structures of Azobenzene Derivatives (**6**—**37**) and Azoxybenzene Derivatives (**38**—**66**)

The substitutents (Rs) are defined in Tables 1 and 2.

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Table 1. Anti-Androgenic Activity of Azobenzene Derivatives

Compound	R	IC_{50}[a] (μM)	LD_{50}[a] (μM)	S-value[a] (LD_{50}/IC_{50})	Binding[a] (% Inhibition)	mp (°C)
2	—	19.3	>100	(>5.2)	57.6	—[b]
6	H	49.3	71.3	1.45	nt[c]	—[b]
7	2,2′-Et	14.3	23.3	1.63	26.8	red oil
8	3,3′-Et	16.9	25.0	1.48	49.2	red oil
9	4,4′-Et	15.3	11.2	0.73	4.8	58—59
10	3,3′-iPr	18.5	19.2	1.04	12.1	red oil
11	4,4′-iPr	16.0	22.9	1.43	<3	111—113
12	2,2′-F	19.8	7.6	0.38	3.6	105—106
13	3,3′-F	16.1	7.4	0.46	<3	72—73
14	4,4′-F	17.0	7.1	0.42	<3	99—101
15	2,2′-Cl	8.7	8.2	0.94	15.0	130—132
16	3,3′-Cl	11.4	3.0	0.26	10.5	100—102
17	4,4′-Cl	>100	>100	—	nt	185—187
18	2,2′-Br	8.4	9.7	1.15	44.8	131—134
19	3,3′-Br	14.1	11.6	0.82	<3	123—125
20	4,4′-Br	>100	>100	—	nt	210—212
21	2,2′-I	7.8	18.7	2.40	56.4	156—159
22	3,3′-I	7.1	12.6	1.77	6.9	153—154
23	4,4′-I	>100	>100	—	nt	243—244
24	3,3′-NO_2	9.4	7.3	0.78	49.6	153—157
25	4,4′-NO_2	9.0	>100	(>11.1)	46.9	228—231
26	2,2′-OAc	5.2	5.5	1.06	53.9	153—155
27	3,3′-OAc	46.9	17.5	0.37	51.4	142—143
28	3,3′-CH_2OAc	>100	>100	—	nt	red oil
29	4,4′-CH_2OAc	8.4	15.4	1.83	nt	134—136
30	2,2′-OH	5.1	5.0	0.99	23.4	—[b]
31	2,2′-OMe	>100	>100	—	nt	152—153
32	3,3′-OMe	21.6	63.0	2.92	nt	90—91
33	4,4′-OMe	8.4	>100	(>11.9)	31.0	160—161
34	2,2′-COOEt	>100	>100	—	nt	81—83
35	3,3′-COOEt	>100	>100	—	nt	107—108
36	4,4′-COOEt	>100	>100	—	nt	142—143
37	2,2′-fused	60.4	>100	(>1.7)	nt	—[b]

a) Values are defined as described in Materials and Methods. *b*) Commercially available compounds. *c*) Not tested.

trast to azobenzene derivatives, the azoxybenzene derivatives prepared existed only in *trans*-form as far as analyzed by ^{1}H-NMR spectroscopy in $CDCl_3$ (no isomerization was observed under the experimental conditions).

Biological Assay The anti-androgenic activity of all the compounds prepared was first evaluated by growth-inhibition assay using the androgen-dependent clonal cell line SC-3, which was derived from Shionogi carcinoma 115,[10] as already reported.[3] Briefly, SC-3 cells (3×10^4 cells/ml in minimum essential medium (MEM) supplemented with 2% v/v fetal bovine serum) were incubated under usual conditions (37 °C, 5% CO_2 atmosphere) with 10 nM testosterone in the presence of various concentration of test compound for three days. After the incubation, the viable cell number was measured by testing the succinate-tetrazolium reductase system using the WST-1 method.[11] The increase of cell number in the absence of only test compound was defined as 100%, and the relative/expedient anti-androgenic activity of test compounds was presented as IC_{50} value (μM) (Tables 1 and 2), *i.e.*, the concentration at which the increase of cell number was reduced to 50%. Of course, the IC_{50} value defined as such includes a contribution from cell toxicity, which is not related to anti-androgenic activity.

For the assessment of the cell toxicity of the compounds, the same assay without addition of testosterone and/or growth-inhibition assay using human promyelocytic leukemia HL-60 cells, which are androgen-independent, was performed. The toxicity was presented as LD_{50} (μM) (Tables 1 and 2), *i.e.*, the concentration at which half of the cells were killed.

The selectivity of the biological activity was assessed in terms of the S-value (Tables 1 and 2), which is defined as LD_{50}/IC_{50}. Superior anti-androgens should show high S-values. Most simply, an S-value of 1 implies that the corresponding compounds elicits SC-3 cell growth inhibition predominantly through cell toxicity, with little contribution of anti-androgenic activity. An S-value of less than 1 might be interpreted as representing partial agonistic/antagonistic androgenic activities of the compounds and/or as a consequence of differences of cell type or culture conditions.

For typical compounds with low IC_{50} values, binding affinity toward nuclear androgen receptor was assessed by competitive binding assay using [^{3}H]-testosterone (Amersham, 95.0 Ci/mmole) based on the reported method.[12—15] Briefly, the nuclear androgen receptor fraction was extracted from SC-3 cells. The collected SC-3 cells were homogenized in a glass-Teflon Potter homogenizer in 3 volumes of 20 mM Tris–HCl (pH 8.0)/0.6 M KCl, and the homogenate was ultracentrifuged (100000 G, 2 h). The supernatant was adjusted to 0.5 mg protein/ml, and was used as the nuclear androgen receptor.[12] This nuclear androgen receptor fraction was incubated with 1 nM [^{3}H]testosterone in the presence of various concentrations of test compound at 4 °C for 16 h. The mixture was fractionated by gel filtration column chromatogra-

Table 2. Anti-Androgenic Activity of Azoxybenzene Derivatives

Compound	R	IC_{50}[a] (μM)	LD_{50}[a] (μM)	S-value[a] (LD_{50}/IC_{50})	Binding[a] (% Inhibition)	mp (°C)
2	—	19.3	>100	(>5.2)	57.6	—[b]
38	H	33.3	17.2	0.52	nt[c]	—[b]
39	2,2′-Et	13.5	17.4	1.29	49.2	Yellow oil
40	3,3′-Et	33.3	7.7	0.23	<3	Yellow oil
41	4,4′-Et	15.3	12.9	0.73	9.4	Yellow oil
42	3,3′-iPr	15.1	20.4	1.35	4.4	Yellow oil
43	4,4′-iPr	15.4	35.6	2.31	<3	Yellow oil
44	2,2′-F	13.5	9.2	0.68	13.8	Yellow oil
45	3,3′-F	15.0	6.7	0.45	<3	48—50
46	4,4′-F	9.2	6.3	0.68	3.6	89—90
47	2,2′-Cl	5.5	>100	(>18.2)	53.0	46—48
48	3,3'-Cl	6.4	6.7	1.05	3.0	96—97
49	4,4′-Cl	>100	>100	—	nt	153—156
50	2,2′-Br	5.2	7.8	1.50	67.8	110—111
51	3,3′-Br	7.8	8.6	1.10	<3	108—109
52	4,4′-Br	>100	>100	—	nt	168—171
53	3,3′-NO_2	31.3	9.3	0.30	nt	152—155
54	2,2′-OAc	5.2	10.3	1.98	67.0	Yellow oil
55	3,3′-OAc	46.9	17.5	0.37	52.8	99—100
56	2,2′-OH	5.1	5.0	0.99	21.1	153—154
57	3,3′-OH	28.4	100.2	3.53	60.0	241—243
58	2,2′-OMe	>100	>100	—	nt	76—77
59	3,3′-OMe	19.7	>100	(>5.1)	16.4	70—71
60	4,4′-OMe	>100	>100	—	nt	—[b]
61	3,3′-CH_2OAc	>100	>100	—	nt	Yellow oil
62	4,4′-CH_2OAc	59.6	>100	(>1.7)	nt	105—107
63	2,2′-COOEt	>100	>100	—	nt	73—75
64	3,3′-COOEt	71.2	70.9	1.00	nt	76—78
65	4,4′-COOEt	>100	>100	—	nt	110—112
66	2,2′-fused	62.7	>100	(>1.6)	nt	136—138

a) Values are defined as described in Materials and Methods. *b*) Commercially available compounds. *c*) Not tested.

phy using Sephadex G25. The protein fraction (high molecular weight, checked by Coomassie Blue staining) was collected, and the radioactivity was measured with a liquid scintillation counter. The values obtained in the absence and presence of 200 nM cold testosterone were defined as 0% inhibition and 100% inhibition, respectively, and the values of % inhibition obtained for 200 nM test compounds are presented in Tables 1 and 2.

The values (IC_{50}, LD_{50}, and value of % inhibition) thus measured showed some deviation from experiment to experiment. However, the order of efficacy of the compounds was reproducible. Therefore, typical set of data are presented.

RESULTS AND DISCUSSION

The anti-androgenic activity of azo- and azoxy-benzene derivatives (**6**—**66**) assessed as described in the experimental section is shown in Tables 1 and 2, respectively, together with that of a typical anti-androgen, flutamide (**2**). As mentioned in the experimental section, a compounds with a low IC_{50} value (μM) and a high S-value can be regarded as superior anti-androgens. In this sense, and in comparison with flutamide (**2**: IC_{50}=19.3 μM, S=>5.2), compounds with an IC_{50} value of below 10 μM and an S-value higher than 10 are more potent than flutamide (**2**); *i.e.*, compounds **25** (IC_{50}=9.0 μM, S=>11.1), **33** (IC_{50}=8.4 μM, S=>11.9), and **47** (IC_{50}= 5.5 μM, S=>18.0). These compounds were shown to bind nuclear androgen receptor with comparable affinity (31.0—53.0% inhibition) to that of flutamide (**2**: 57.6% inhibition). Compound **59** showed anti-androgenic activity (IC_{50}= 19.7 μM, S=>5.1) comparable to that of flutamide (**2**), but its affinity to nuclear androgen receptor was rather low (16.4% inhibition). The poor correlation between anti-androgenic SC-3 growth-inhibiting activity (IC_{50} value) and affinity for nuclear androgen receptor (value of % inhibition) of the compounds mentioned above might be interpreted in terms of differences in the uptake (cell/nuclear membrane permeability) of the compounds.

Some compounds (**7**—**11**, **13**—**16**, **18**, **19**, **21**, **22**, **24**, **26**, **29**, **30**, **39**, **41**—**46**, **48**, **50**, **51**, **54**, **56**) showed IC_{50} values lower than that of flutamide (**2**), but they were also more toxic than flutamide (**2**) with S-values of less than 3. Though these compounds are toxic, some of them possess high (**50**, **54**, **57**: 60—68% inhibition) or moderate (**8**, **18**, **21**, **24**, **26**, **27**, **39**, **55**: 44—57% inhibition) affinity for the receptor, suggesting they may be useful tools for investigating the nuclear androgen receptor.

Concerning the structure–activity relationships of azo- and azoxy-benzene derivatives, we may make the following generalizations, based mainly on the IC_{50} values, though there are some exceptions and it is difficult to reach clear-cut conclusions.

(1) Generally, azobenzene derivatives possess activity similar to the corresponding azoxybenzene derivatives [except for the 4,4′-dimethoxy analogs (**33** and **60**)], with the latter being slightly more active (exceptions are **8**/**40**, **24**/**53** and **37**/**66**).

(2) Concerning the halogenated compounds, the activity

generally decreased in the order of 2,2′- >3,3′- >4,4′-substitution, and 4,4′-dihalogenated analogs are inactive (both IC_{50} and LD_{50} values are >100 μM) (the exceptions are difluorinated compounds and **21**/**22**). 2,2′-Dichlorinated azoxybenzene (**47**) is one of the superior anti-androgens identified in this work. The difluorinated and active dihalogenated compounds are highly toxic with S-values of less than 1.

(3) The activity order of 2,2′- >3,3′- >4,4′-substitution mentioned above is reversed for the dimethoxy-substituted azobenzenes (**31**—**33**), with the 4,4′-dimethoxy analog (**33**) being another of the superior anti-androgens prepared in this work. A further potent anti-androgen is the 4,4′-dinitro analog of azobenzene (**25**).

(4) The effects of substituents on the activity are different depending on the position of substitution. The activity roughly decreased in the order of OH≅OAc>I>Br≅Cl> Et≅F≫OMe/COOEt (inactive) for 2,2′-substitution, I> NO_2>Cl>Br>F/Et/iPr/OH/OMe > OAc/COOEt for 3,3′-substitution, and NO_2>F/Et/iPr≫Cl/Br/I/COOEt (inactive) for 4,4′-substituion. The effects of 4,4′-dimethoxy and 4,4′-acetoxymethyl substitution are quite different, *i.e.*, the 4,4′-dimethoxy analog of azobenzene (**33**) is a potent anti-androgen as already mentioned, while the corresponding azoxybenzene analog (**60**) is completely inactive. Similarly, the 4,4′-acetoxymethyl analog of azobenzene (**29**) is potent, while the corresponding azoxybenzene analog (**62**) has weak activity.

Although clear-cut structure-activity relationships could not be obtained, our results indicated that azo- and azoxybenzene could be available as structural frameworks for non-steroidal anti-androgens. As azo- and azoxy-benzene skeletons have also been utilized for the structural development of ligands for another nuclear receptor, retinoic acid receptors,[9] they might also be applicable in the development of ligands for other nuclear receptors.

In conclusion, we prepared a series of novel non-steroidal anti-androgens based on azo- and azoxy-benzene skeletons. Among them, **25**, **33** and **47** are superior anti-androgens, more potent than flutamide (**2**). Further structural development studies and further biochemical/biological studies of these anti-androgens are planned.

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