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Anti-Androgens with Full Antagonistic Activity Toward Human Prostate Tumor LNCaP Cells with Mutated Androgen Receptor

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Abstract—Anti-androgens were designed based on the principle of inhibiting the folding of helix 12 of the nuclear androgen receptor. The prepared anti-androgens exhibited full antagonistic activity toward human prostate tumor LNCaP cells with T877A point-mutated nuclear androgen receptor, as far as examined, towards which other known anti-androgens, including hydroxyflutamide, are inactive or act as androgen agonists.

Androgens, typically testosterone, are endogenous ligands for the nuclear androgen receptor (AR) which is a member of the steroid/thyroid/retinoid/vitamin D_3 nuclear receptor superfamily, and play diverse physiological and pathophysiological roles.[1,2] AR, like other nuclear receptors, consists of three main functional domains including the ligand-binding domain (LBD), DNA-binding domain (DBD) and amino-terminal domain (A/B region).[3–5] 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. Among the substructures, the helix H12 plays a critical role in the ligand-dependent activation of the receptor, that is, it 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.[6,7]

Among the pathophysiological effects elicited by androgens, a role as endogenous tumor promoters, especially for prostate tumor, is well known. Because of this, anti-androgens (AA), which antagonize endogenous androgens by competitively binding to AR, are expected to be effective for treatment of androgen-dependent tumors, especially for prostate tumor.[2] On the basis of a mechanistic consideration of AR activation, we can consider two types of AAs. One is AAs which induce misfolding of helix 12 (denoted as misfolding inducers in this paper), and the other is AAs which inhibit the folding of helix 12 (denoted as folding inhibitors in this paper) (Fig. 1). Our computer-assisted docking studies using the X-ray structure of the LBD of AR suggested that all currently known AAs, including clinically useful flutamide and its active form, hydroxyflutamide, can be categorized as misfolding inducers.

The major obstacle in the treatment of prostate tumor with AAs is the sudden appearance of AA-resistant cells. These cells have generally lost androgen-dependency in their growth and, further, AAs promote their growth, whereas AAs had suppressed their growth before the cells acquired AA-resistance. One major molecular mechanism of AA-resistance is point mutation of AR. Some point mutations, including T877A and T874H, are clinically established.[8,9] ARs which possess such a point mutation are considered to take a helix 12-folded conformation, and are constitutively active even in the absence of the cognate ligand, androgen (Fig. 1). Of course, these mutated ARs can bind androgens and misfolding inducer-type AAs, which stabilize the active conformation of the AR, leading to super-activation of the mutated ARs. Therefore, to overcome the problem of AA-resistance based on AR mutation, other types of AAs, which bind to the mutated ARs and induce unfolding (or inhibit the folding of) helix 12, would be useful (Fig. 1).

In this paper, we describe the design and biological activities of AAs with an isoxazolone moiety (**1**–**4**, Fig. 2)

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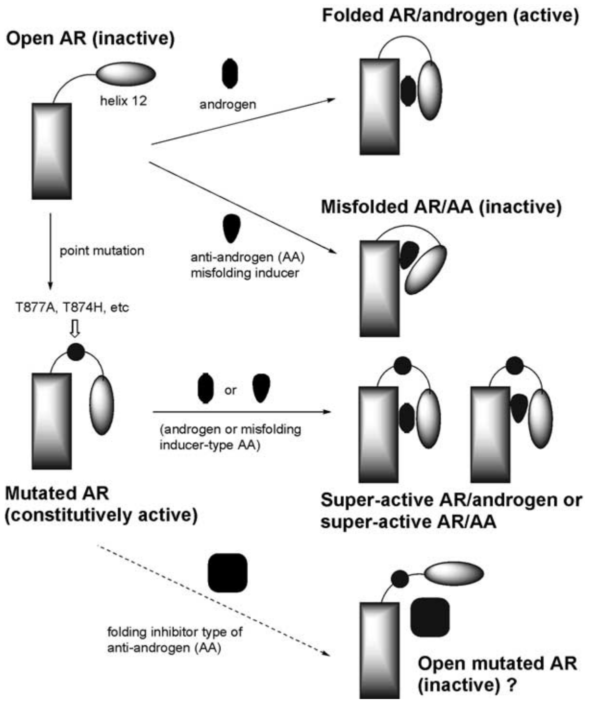


Figure 1. Schematic illustration of putative mechanisms of activation and inactivation of ARs by point mutation and with AAs.

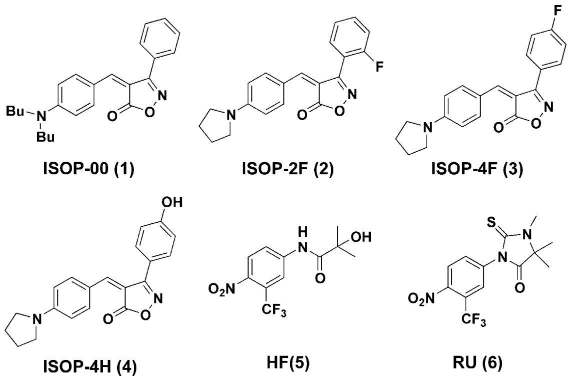


Figure 2. Structures of folding inhibitor-type AAs (**1**–**4**) and known AAs [hydroxyflutamide: HF (**5**) and RU (**6**)].

which act as possibly full antagonists in human prostate tumor LNCaP cells with T877A mutation in the AR, towards which currently known AAs act as androgen agonists.

Design of Folding Inhibitor-type AAs

Recently, we reported novel structural types of AAs with an isoxazolone skeleton.[10–12] Computer-assisted docking studies suggested that (*Z*)-4-(4-*N*,*N*-diethylaminophenylmethylene)-3-methyl-5(4*H*)-isoxazolone can be a ligand for AR, with the 3-methyl moiety interacting with helix 12 of AR. Based on this result, we considered that by the use of the isoxazolone skeleton as a scaffold, folding inhibitor-types AAs might be prepared by replacing the 3-methyl group with a bulky group. This consideration, as well as our previous studies on nuclear receptor ligands,[4,5,7,10–12] led us to design 3-phenylisoxazolone derivatives **1**–**4** (Fig. 2).

Compounds **1**–**4** were prepared by usual synthetic methods as illustrated in Figure 3, and the structures were confirmed by NMR and mass spectrometry, and by appropriate analytical values.[13–16]

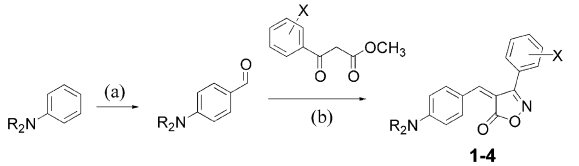
Biological Activities of Compounds 1–4

Preliminary evaluation of androgenic and anti-androgenic activities, that is, activities toward normal ARs, of the compounds was done 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.[12,17–19] The ability of the compounds to be ligands for AR is indicated by the former assay, while the latter assay determines whether the compounds are androgen agonists or antagonists.

For receptor-binding assay, we adopted competitive binding assay using [^{3}H]testosterone and recombinant human AR as described previously.[12,19] The recombinant AR was prepared from cytosol of *Escherichia coli* transformed with a human AR LBD expression vector (GST-hARLBD), which codes amino acids 627–919 of hAR fused with GST protein under the *lac* promoter.[12,19] 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. 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 thus obtained was 1.3 μM. The values of relative receptor-binding activity of the test compounds, that is, (K_i value of flutamide)/(Ki value of test compound), were in the range of 100–150 (details will be published elsewhere). In the growth promotion/inhibition assay using androgen-dependent SC-3 cells, none of the compounds prepared showed growth-promoting activity, suggesting that none was an androgen agonist. All the compounds prepared showed dose-dependent growth-inhibitory activity on SC-3 cells incubated in the presence of 10 nM testosterone, suggesting that all the compounds act as AAs, at least toward normal AR.

Activity of compounds **1**–**4** toward mutated, constitutively active ARs was analyzed by the use of LNCaP and 22Rv1 cells, which express T877A and T874H point-mutated ARs, respectively.[8,9] All of the compounds prepared showed growth-inhibitory activity on these cells, while known classical AAs, hydroxyflutamide (HF: **5**, Fig. 2) and RU (**6**, Fig. 2) showed growth-promoting activity (data for LNCaP cells were shown in Fig. 4a, bottom). The result suggests that compounds **1–4** do not elicit androgenic activity (do not super-activate the mutated ARs) and act as AAs on LNCaP and 22Rv1 cells, while HF (**5**) and RU (**6**) act as androgen agonists on these cells.

Quantitative assessment of androgenic and anti-androgenic activities of the compounds was performed by measurement of the amount of prostate-specific antigen (PSA) produced by LNCaP cells (Fig. 4). PSA is a marker molecule of prostate tumor malignancy and its production is known to be induced by androgens.[20,21] Therefore, androgenic activity can be detected in terms of enhanced production of PSA (Fig. 4a), and anti-androgenic activity can be assessed in terms of inhibition of PSA production induced by androgens (Fig. 4b).



1-4

Figure 3. Synthetic procedures of compounds **1–4**: (a) $POCl_3$, DMF, 80 °C, 2 h; (b) $NH_2OH{\cdot}HCl$, phosphoric acid, rt, overnight.

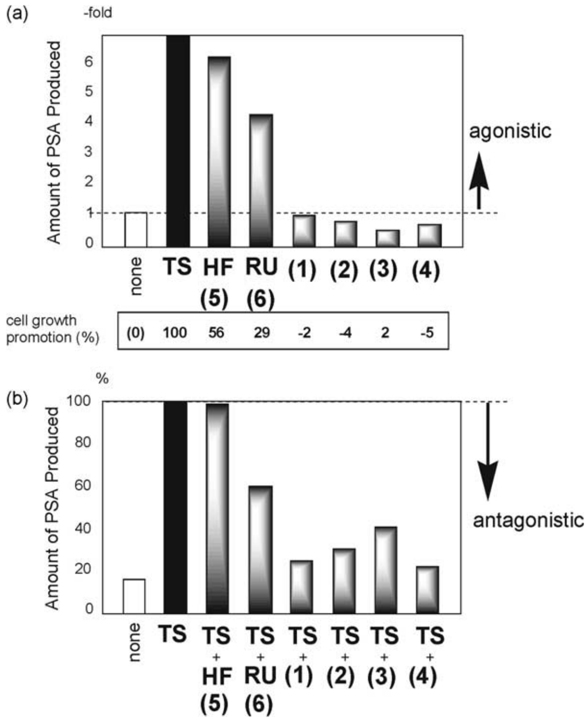


Figure 4. Effects of prepared AAs (**1–4**) and known AAs [hydroxyflutamide: HF (**5**) and RU (**6**)] on PSA production by LNCaP cells. (A) Amount of PSA produced in the absence or presence of testosterone (TS: 10 nM) or test compounds (100 nM). The amount of PSA produced by non-treated cells was defined as 1-fold. Values below the panel indicate cell growth promoting activity (%) of the compounds when the activity of TS was defined as 100%. (B) Effect of test compounds (300 nM) on the amount of PSA produced in the presence of 10 nM testosterone (TS). The amount of PSA produced in the presence of 10 nM testosterone (TS) alone was defined as 100%.

The amount of PSA was measured by the use of an ELISA PSA Assay Kit (Cayman Co. Ltd) according to the protocol recommended by the supplier. The experiment was performed in triplicate and repeated at least two times. Though the values deviated from experiment to experiment, the results (order of potency) were basically reproducible, and a typical set of data is shown in Figure 4.

As shown in Figure 4, LNCaP cells incubated under the normal conditions produced a small amount of PSA (the amount was defined as 1-fold in Fig. 4a), and addition of 10 nM testosterone (TS) markedly enhanced the production (the amount was defined as 100% in Fig. 4b). Classical AAs, HF (**5**) and RU (**6**), also enhanced the production to 4–6 times (Fig. 4a), suggesting that these AAs act as androgen agonists toward LNCaP cells, which is in accordance with the cell growth promotion/inhibition assay data for LNCaP and 22Rv1 described above. Compounds **1–4** did not enhance the PSA production at all in the concentration range of 10–1000 nM, suggesting that these compounds possess no androgenic activity in the concentration range examined (Fig. 4a). In the inhibition assay of testosterone-induced PSA production (Fig. 4b), HF (**5**) was almost completely inactive (98%), and RU (**6**) showed moderate inhibitory activity (58%). The results suggest that HF (**5**) and RU (**6**) act as a full agonist and a partial agonist/antagonist toward LNCaP cells in these assay systems, respectively. In contrast, all of compounds **1–4** showed inhibitory activity on testosterone-induced PSA production (Fig. 4b). This result, combined with the results in Fig. 4a, suggests that the compounds act as possibly full AAs even on the LNCaP cells, which constitutively express active mutated AR. Among the compounds prepared, the 4-hydroxyl analogue (**4**) showed the most potent inhibitory activity on PSA production. No substituent effect at the 3-phenyl moiety of the isoxazolone derivatives on the activity was apparent, as far as compared among compounds **1–4**. Further investigation is in progress.

Conclusion

We designed AAs **1–4** as candidate inhibitors of the folding of helix 12 of AR, with the aime of obtaining full antagonistic activity toward constitutively active point-mutated ARs. The prepared compounds **1–4** were indeed found to act as full AAs on human prostate tumor LNCaP cells expressing T877A mutated AR, as far as examined, while classical and clinically used AAs act as full androgen agonists or partial androgen agonists/antagonists on these cells.

Acknowledgements

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References and Notes

1. Mooradian, A. D.; Morley, J. E.; Korenman, S. G. *Endocr. Rev.* **1987**, *8*, 1.

2. Zhi, L.; Martinborough, E. *Ann. Rep. Med. Chem.* **2001**, *36*, 169.

3. MacLean, I. J.; Warne, G. L.; Zajac, J. D. *J. Steroid Biochem. Mol. Biol.* **1997**, *62*, 233.

4. Hashimoto, Y.; Shudo, K. *Cell Biol. Rev.* **1991**, *25*, 209.

5. Hashimoto, Y. *Cell Struct. Funct.* **1991**, *16*, 113.

6. Wurtz, J.; Bourguet, W.; Renaud, J.; Vivat, V.; Chambon, P.; Moras, D.; Gronemeyer, H. *Nat. Struct. Biol.* **1996**, *3*, 87.

7. Eyrolles, L.; Kawachi, E.; Matsushima, Y.; Nakajima, O.; Kagechika, H.; Hashimoto, Y.; Shudo, K. *Med. Chem. Res.* **1992**, *2*, 361.

8. Beer, H.-D.; Florence, C.; Dammeier, J.; McGuire, L.; Werner, S.; Duan, D. R. *Oncogene* **1997**, *15*, 2211.

9. Sramkoski, R. M.; Pretlow, T. G.; Giaconia, J. M.; Pretlow, T. P.; Schwartz, S.; Sy, M. S.; Marengo, S. R.; Rhim, J. S.; Zhang, D. S.; Jacobberger, J. W. *In Vitro Cell. Dev. Biol. Anim* **1999**, *35*, 403.

10. Hashimoto, Y. *Bioorg. Med. Chem.* **2002**, *10*, 461.

11. Hashimoto, Y. *Mini. Rev. Med. Chem.* **2002**, *2*, 543.

12. Ishioka, T.; Kubo, A.; Koiso, Y.; Nagasawa, K.; Itai, A.; Hashimoto, Y. *Bioorg. Med. Chem.* **2002**, *10*, 1555.

13. (*Z*)-4-(4-Pyrrolidinophenylmethylene)-3-phenyl-5(4*H*)-isoxazolone (ISOP-00: **1**): 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: 8.42 (brs, 2H), 7.50–7.60 (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.

14. (*Z*)-4-(4-Pyrrolidinophenylmethylene)-3-(2-fluoro-phenyl)-5(4*H*)-isoxazolone (ISOP-2F: **2**): red needles [hexane/EtOAc]; mp 200–201 °C; MS(FAB) *m/z* 337 (MH^+); ^1H NMR (500 MHz, $CDCl_3$) δ: 8.41 (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.

15. (*Z*)-4-(4-Pyrrolidinophenylmethylene)-3-(4-fluoro-phenyl)-5(4*H*)-isoxazolone (ISOP-4F: **3**): purple crystals [hexane/EtOAc]; mp 217 °C; MS(FAB) *m/z* 337 (MH^+); ^1H NMR (500 MHz, $CDCl_3$) δ: 8.40 (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.

16. (*Z*)-4-(4-Pyrrolidinophenylmethylene)-3-(4-hydroxyphenyl)-5(4*H*)-isoxazolone (ISOP-4H: **4**): red crystals [CH_2Cl_2]; mp 234–235 °C; MS(FAB) *m/z* 335 (MH^+); ^1H NMR (500 MHz, DMSO-d_6) δ: 9.98 (s, 1H), 8.43 (brd, $J = 9.0$ Hz, 2H), 7.45 (s, 1H), 7.44 (d, $J = 8.0$ Hz, 2H), 6.93 (d, $J = 8.0$ Hz, 2H), 6.70 (d, $J = 9.0$ Hz, 2H), 3.46 (td, $J = 3.5$, 3.5 Hz, 4H), 1.98 (tt, $J = 3.5$, 3.5 Hz, 4H), HRMS (FAB, MH^+) $C_{20}H_{19}N_2O_3$, calcd for 335.1396, found 335.1432.

17. Hiraoka, D.; Nakamura, N.; Nishizawa, Y.; Uchida, N.; Noguchi, S.; Matsumoto, K.; Sato, B. *Cancer Res.* **1987**, *47*, 6560.

18. Miyachi, H.; Azuma, A.; Kitamoto, T.; Hayashi, K.; Kato, S.; Koga, M.; Sato, B.; Hashimoto, Y. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1483.

19. Takahashi, H.; Ishioka, T.; Koiso, Y.; Sodeoka, M.; Hashimoto, Y. *Biol. Pharm. Bull.* **2000**, *23*, 1387.

20. Lee, C.; Sutkowski, D. M.; Sensibar, J. A.; Zelner, D.; Kim, I.; Amsel, I.; Shaw, N.; Prins, G. S.; Kozlowski, J. M. *Endocrinology* **1995**, *136*, 796.

21. Riegman, P. H. J.; Vlietstra, R. J.; van der Korput, H. A. G. M.; Brinkmann, A. O.; Trapman, J. *Mol. Endocrinol.* **1991**, *5*, 1921.