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Review Article

Thalidomide as a multitarget drug and its application as a template for drug design

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CONTENTS

Abstract

Thalidomide is a hypnotic/sedative drug which was withdrawn from the market because of teratogenicity. However, it has been established to be useful for the treatment of various diseases, including Hansen's disease and various cancers. Thalidomide elicits a wide range of pharmacological effects, including anticachexia, antitumor-promoting, antiangiogenic, immunosuppressing, antiviral, hypoglycemic, cell differentiation-inducing and antimetastatic activities. It appears to be a multitarget drug, and hypothetical target events/molecules of thalidomide include TNF-α production, nuclear androgen receptor, cyclooxygenases, nuclear retinoic acid receptor, aminopeptidases and α-glucosidase. Specific and potent compounds acting on each of these target phenomena/molecules have been prepared by appropriate modification of the thalidomide structure and are expected to be superior lead compounds for novel immunomodulators, antiangiogenic agents and antitumor-promoting agents.

Introduction

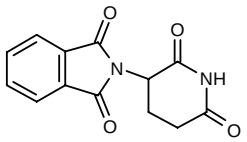
Thalidomide is a hypnotic/sedative drug which was launched in the 1950s, but was withdrawn from the market because of severe teratogenicity in the 1960s (1-3). However, thalidomide has been established to be useful for the treatment of Hansen's disease, and the drug was formally approved for this indication by the U.S. Food and Drug Administration in 1998 under critical control. Many reports have appeared on its therapeutic usefulness in various diseases, including several cancers, rheumatoid arthritis, graft-*versus*-host disease, AIDS and others (1-3). Although pharmacological applications of thalidomide have been widely investigated, the molecular basis of its actions has not yet been clarified. The beneficial pharmacological effects elicited by thalidomide include anticachexia, antiinflammatory, antitumor-promoting, antiangiogenic, tumor cell invasion-inhibiting, antiviral and hypoglycemic effects (Table I).

The prevailing hypothesis had been that all of the beneficial effects of thalidomide are elicited through regulation of tumor necrosis factor-α (TNF-α) production. However, our structural development studies of thalidomide suggested that thalidomide is a multitarget drug. This implied possible usefulness of thalidomide as a template for development of various kinds of drugs, and we hypothesized that there might be individual target phenomena/molecules for each pharmacological effect (Table I). Our studies on the development of specific and potent compounds for each of these target phenomena/molecules are reviewed.

Tumor necrosis factor-α production regulators

Our studies on the TNF-α production-regulating activity of thalidomide revealed that the effect elicited by the drug is bidirectional, depending on both the cell type and the cell stimulator (1-8). For example, thalidomide acts as a TNF-α production enhancer on the human leukemia cell line HL-60 when the cells are stimulated with tetradecanoylphorbol 13-acetate (TPA). However, it acts as a TNF-α production inhibitor on the same cell line and in the same concentration range when okadaic acid is used as a stimulator. With another type of human leukemia cell line, THP-1, thalidomide acts as a TNF-α production inhibitor regardless of the stimulator used. Studies on the structural development of

Table I: Typical pharmacological effects elicited by thalidomide and their putative/hypothetical target phenomena/molecules in relation to cancer chemotherapy.



Pharmacological effect	Putative/hypothetical target phenomena/molecules
Anticachexia	TNF-α production inhibition
Antitumor-promoting	TNF-α production inhibition, nuclear androgen receptor antagonist, nuclear estrogen receptor antagonist, nuclear retinoic acid receptor agonist, COX-2 inhibition
Antiinflammatory	TNF-α production inhibition, COX inhibition, NO synthase inhibition
Antiangiogenic	Thymidine phosphorylase/platelet-derived endothelial cell growth factor (TP/PD-ECGF) inhibition, TNF-α production inhibition
Anti-cell invasion	Puromycin-sensitive aminopeptidase (PSA) inhibition
Antiviral	Dipeptidylpeptidase type IV (DPP-IV) inhibition, α-glucosidase inhibition, TNF-α production inhibition
Hypoglycemic	α-Glucosidase inhibition, TNF-α production inhibition

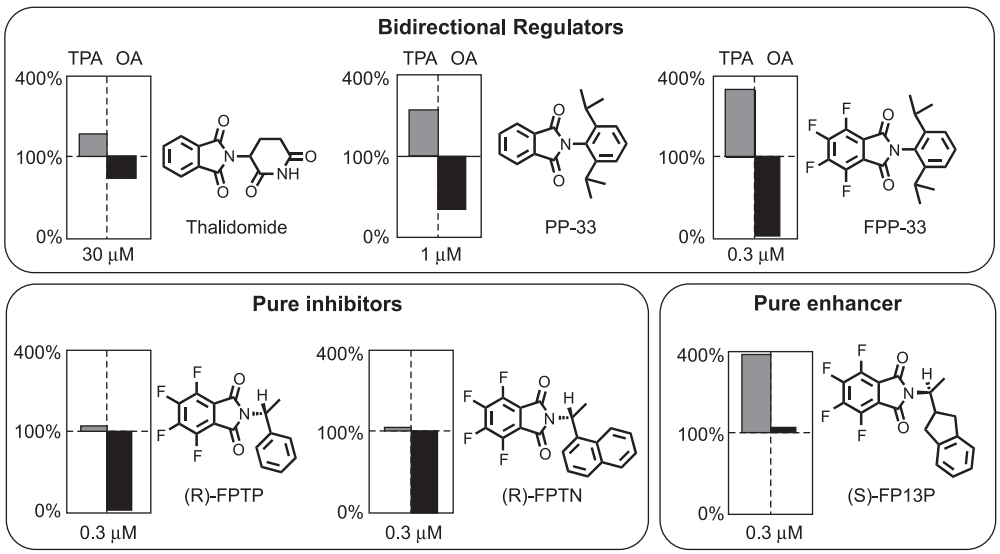


Fig. 1. Typical TNF-α production regulators. Structures of thalidomide and our typical TNF-α production regulators are shown with their TNF-α production-regulating activity. TNF-α production-enhancing and -inhibiting activities of each compound at the concentration described under each panel are shown as left and right bars, respectively, in each panel. HL-60 cells were treated with 10 nM TPA (for assessment of TNF-α production-enhancing activity) or 50 nM OA (for assessment of TNF-α production-inhibiting activity) in the presence of test compound [30 μM for thalidomide, 1 μM for PP-33 and 0.3 μM for FPP-33, (*R*)-FPTP, (*R*)-FPTN and (*S*)-FP13P]. The amount of TNF-α produced in the presence of 10 nM TPA alone or 50 nM OA alone was defined as 100%.

thalidomide afforded highly potent bidirectional TNF-α production regulators (*e.g.*, PP-33 and FPP-33) and complete separation of the directionality, *i.e.*, pure inhibitors (*e.g.*, (*R*)-FPTP and (*R*)-FPTN) and pure enhancers (*e.g.*, (*S*)-FP13P) (Fig. 1) (1-3, 8-11). The planar structure-activity relationships (SARs) are similar for TNF-α production enhancement and inhibition, *i.e.*, FPP-33 is more potent than PP-33 in both TNF-α production enhancement and inhibition. The introduction of an asymmetric carbon unit between the phthalimide nitrogen atom and an aromatic moiety made it possible to separate the directionality, *i.e.*, (*R*)-forms are generally potent in TNF-α production inhibition and weak in TNF-α production enhancement compared with the corresponding (*S*)-forms. Some of our bidirectional TNF-α production regulators and inhibitors prolonged the life span of mice with cachexia induced by lipopolysaccharide injection.

Antiangiogenic agents

Ongoing phase II/III clinical studies of thalidomide as an antiangiogenic agent prompted us to assess the antiangiogenic activity of our compounds. Some of our

TNF-α production regulators, especially (*R*)-FPTP (Fig. 1), showed more potent antiangiogenic activity than thalidomide at a much lower dose (12). Although we were able to develop potent antiangiogenic agents based on TNF-α production-regulating activity, including (*R*)-FPTP, the SARs in an antiangiogenic assay and TNF-α production-regulating assay are not well correlated. Moreover, SAR studies for other biological assay systems in which thalidomide is effective indicated that the pharmacological effects of thalidomide cannot be attributed to its TNF-α production-regulating activity alone. These results led us to consider structural modifications of thalidomide based on different target molecules/phenomena (other than TNF-α), which are considered to be related to the above-mentioned pharma- cological effects elicited by thalidomide. In the case of antiangiogenic activity, we proposed thymidine phosphorylase (TP)/platelet-derived endothelial cell growth factor (PD-ECGF) as a putative target molecule (Table I).

Our structural development study targeting inhibition of TP/PD-ECGF activity yielded several homophthalimide analogs, including NPIQ-22 (Fig. 2), which showed more potent TP/PD-ECGF inhibitory activity than the classical inhibitor 5-nitrouracil (Fig. 2) (13, 14). NPIQ-22 and related inhibitors are considered to be lead compounds for the development of novel type(s) of TP/PD-ECGF inhibitors.

Androgen antagonists

TNF-α is reported to be one of the endogenous tumor promoters. A preliminary study indicated that some of our TNF-α production regulators show moderate antitumor-promoting activity, although the SARs in an antitumor-promoting assay and TNF-α production-regulating assay were not directly correlated. To develop more potent antitumor-promoting agents, we focused on another endogenous tumor promoter, *i.e.*, fibroblast growth factor 10 (FGF-10). FGF-10 is reported to act as a tumor promoter, especially in prostate cancer, and its production is induced by androgen. Considering the effectiveness of thalidomide in the treatment of prostate cancer and its structural similarity to a classical androgen antagonist, DIMP (Fig. 3), we anticipated that superior nonsteroidal androgen antagonists could be prepared by structural development of thalidomide.

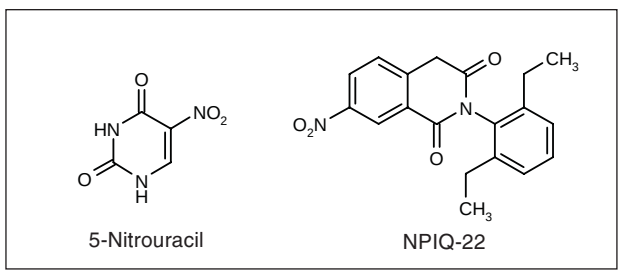


Fig. 2. Typical thymidine phosphorylase/platelet-derived endothelial cell growth factor inhibitors.

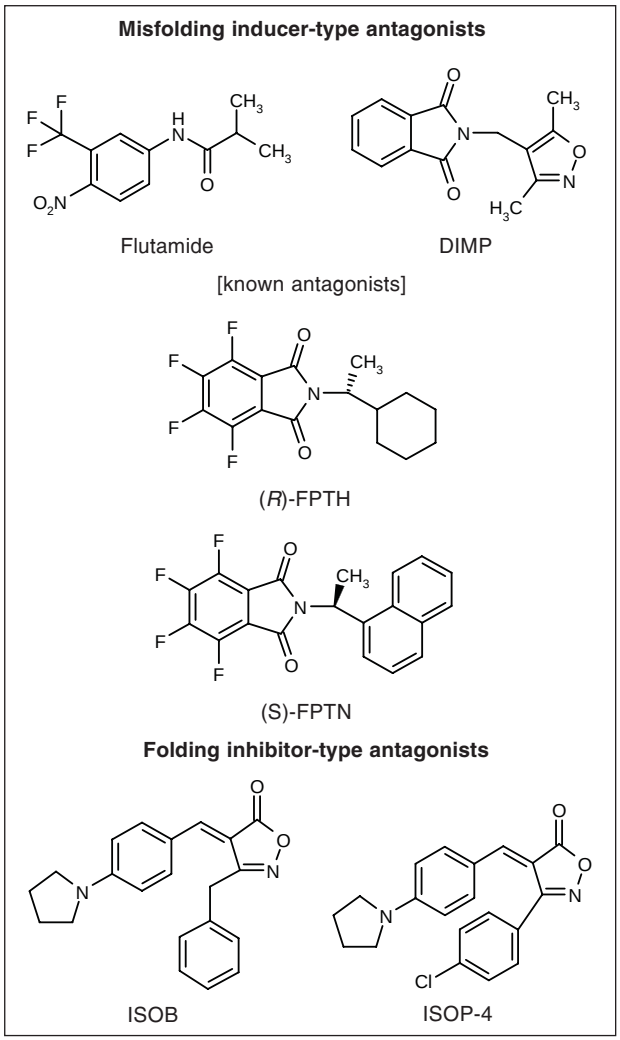


Fig. 3. Typical androgen antagonists.

Androgens, typically testosterone and its active metabolite 5α-dihydrotestosterone, elicit their biological activity by binding to and activating a specific receptor, nuclear androgen receptor (AR), and play diverse physiological and pathophysiological roles. Among the pathophysiological effects elicited by androgens, a role as endogenous tumor promoters, especially for prostate tumors, is well known. Because of this, androgen antagonists, which antagonize endogenous androgens by competitively binding to the AR, are expected to be effective for treatment of androgen-dependent tumors, especially prostate tumors.

The androgen receptor is a member of the steroid/retinoid/thyroid/vitamin D_3 nuclear receptor superfamily and is a ligand-dependent specific transcription factor. Like other nuclear receptors, the AR consists of three main functional domains including the ligand-binding domain (LBD), DNA-binding domain (DBD) and amino-terminal domain (A/B region). The general structure of the LBD has been elucidated by X-ray

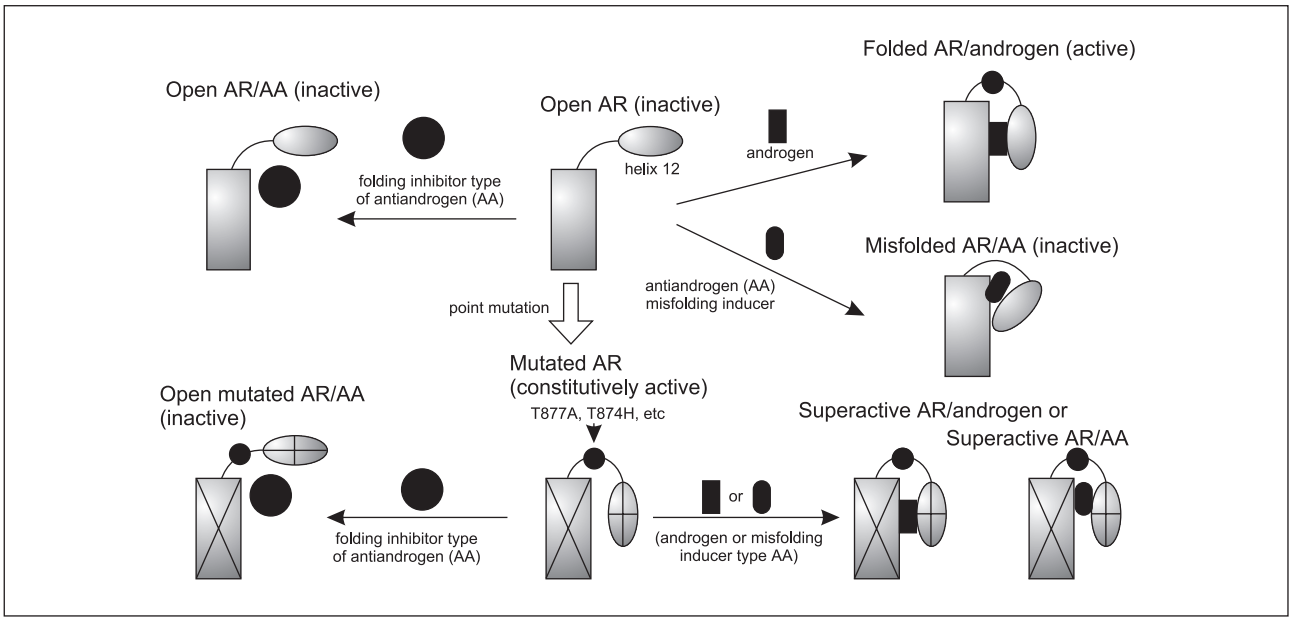


Fig. 4. Schematic illustration of putative mechanisms of activation and inactivation of androgen receptors (AR) by point mutation and with androgen antagonists (AA).

crystallography to be composed of 12 α-helices and a small β-sheet. Among the substructures, the helix H12 plays a critical role in the ligand-dependent activation of the receptor, *i.e.*, 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 (Fig. 4).

Structural development studies of thalidomide based on androgen-antagonist activity afforded several compounds showing much more potent antiandrogenic activity than flutamide (Fig. 3), which had been widely used for the treatment of prostate cancer before a superior androgen antagonist, bicalutamide, appeared [*e.g.*, (*S*)-FPTN and (*R*)-FPTH] (Fig. 3) (1-3, 15). Generally speaking, antagonists for nuclear receptors can be classified into two categories. One comprises antagonists which induce misfolding of helix 12 (denoted as misfolding inducers), and the other antagonists which inhibit the folding of helix 12 (denoted as folding inhibitors) (Fig. 4). Our computer-assisted docking studies using the X-ray structure of the LBD of the AR suggested that all currently known androgen antagonists, including clinically useful flutamide, as well as (*S*)-FPTN and (*R*)-FPTH (Fig. 3), can be categorized as misfolding inducers.

The major obstacle in the treatment of prostate tumors with androgen antagonists is the sudden appearance of antiandrogen-resistant cells. These cells have generally lost androgen dependency in their growth and, furthermore, androgen antagonists promote their growth despite having suppressed growth before the cells acquired resistance. One major molecular mechanism of the resistance is point mutation of the AR. Some point mutations, including T877A and T874H, are clinically established. ARs which possess such a point mutation are considered to take a helical 12-folded conformation and are constitutively active even in the absence of the cognate ligand androgen (Fig. 4). Of course, these mutated ARs can bind androgens and misfolding inducer-type androgen antagonists, which stabilize the active conformation of the AR, leading to superactivation of the mutated ARs. Therefore, to overcome the problem of antiandrogen resistance based on AR mutation, different types of androgen antagonists other than misfolding inducers, which bind to the mutated ARs and induce unfolding (or inhibit the folding) of helix 12, would be useful (Fig. 4).

Therefore, further structural development assisted by computer docking studies using the three-dimensional structure of AR was performed. These studies provided only misfolding inducers, because the coordinates of the LBD of AR with closed conformation were used as a template. Therefore, we planned to introduce a bulky group into a candidate structure derived from the computer-assisted docking study, at the position where helix 12 interacts. This strategy afforded isoxazolone-type compounds, including ISOP-4 and ISOB (Fig. 3) (3, 16). Evaluation of AR binding affinity showed that ISOP-4 and ISOB bind AR with an affinity 220-fold and 214-fold higher than that of flutamide, respectively. Moreover, as expected, these nonsteroidal/nonanilide-type androgen antagonists are active on the human prostate tumor cell line LNCaP, which is resistant to known androgen antagonists, including flutamide and bicalutamide, possibly because of its point-mutated aberrant AR (17).

Cyclooxygenase inhibitors

Another possible target for antitumor-promoting activity is cyclooxygenase (COX). COX is an enzyme which catalyzes the synthesis of prostaglandins from arachidonic acid and is well known as a target molecule of nonsteroidal antiinflammatory drugs, including aspirin. There are two isoforms and one variant of COX, *i.e.*, COX-1, COX-2 and COX-3. COX-1 is constitutively expressed in most tissues, whereas COX-2 is inducible. The third one, COX-3, is a variant of COX-1. Overexpression of COX-2 has been detected in various tumors and its role in carcinogenesis and angiogenesis has been well documented. As such, COX-2 has been suggested to be an important pharmacological target for the prevention and treatment of cancer. COX-2 inhibitors, including celecoxib and sulindac, have been considered for chemoprevention of various cancers, including colon and prostate cancers. Although thalidomide is known to suppress lipopolysaccharide-induced expression of COX-2, the direct effect of the drug on COX had not been established. Our study showed that thalidomide itself possesses COX-1/2-inhibiting activity with a potency comparable to that of aspirin (18). Introduction of a methyl group at the asymmetric carbon of thalidomide in (*R*)-form, *i.e.*, (*R*)-methyl-thalidomide, resulted in enhancement of the activity, with higher efficacy for COX-2 inhibition (Fig. 5). The corresponding (*S*)-isomer is completely inactive. Introduction of a substitutent into the phthalimide aromatic ring of (*R*)-methylthalidomide modulates COX-1/2 selectivity, *i.e.*, introduction of an electron-withdrawing group at the 6-position (*meta*) or introduction of an electron-donating group at the 5-position (*ortho*) makes the compound COX-2-selective, and introduction of an electron-withdrawing group at the 5-position or introduction of an electron-donating group at the 6-position makes the compound COX-1-selective (18). Structural development studies resulted in inhibitors with various COX-1/2 selectivities, for example, AIO-0101, a COX-1-selective inhibitor, UP-00, a COX-2-selective inhibitor, and NI-0101, a nonselective inhibitor (Fig. 5) (3, 18, 19).

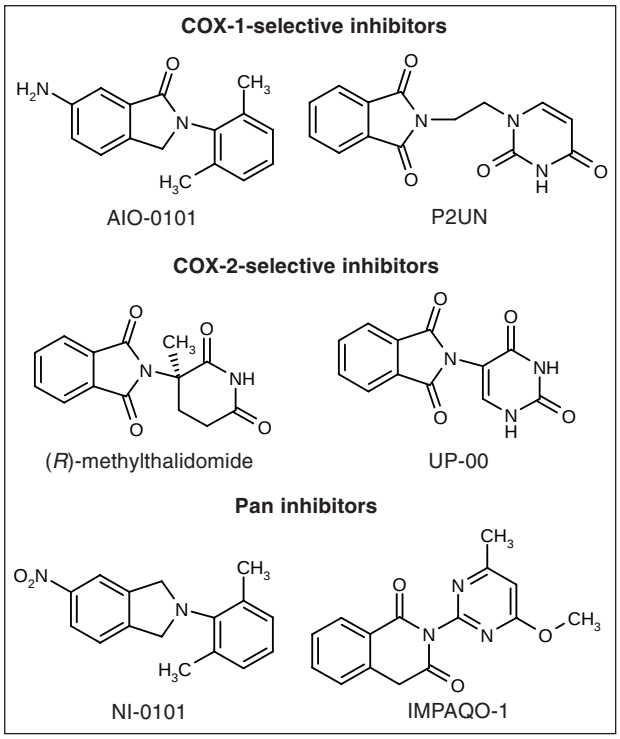


Fig. 5. Typical COX inhibitors.

Tumor cell invasion inhibitors

Concerning anti-cell invasion activity, we focused on aminopeptidase-inhibitory activity. Our structural development studies of thalidomide resulted in specific puromycin-sensitive aminopeptidase (PSA) inhibitors (*e.g.*, PIQ-22 and PAQ-22) (Fig. 6) (3, 13, 20-24). Historically, these inhibitors were developed as aminopeptidase N (APN) inhibitors based on a biological assay measuring the hydrolysis of L-alanine 7-amino-4-methylcoumaryl-7-amide using human lymphoblastic leukemia MOLT-4 cell cultures (20-22). However, PIQ-22 and PAQ-22 were shown to be inactive against APN, which led us to identify the target molecule of these inhibitors. Isolation and amino acid sequencing indicated that PSA is the enzyme which is specifically inhibited by our homophthalimide-type aminopeptidase inhibitors (23).

Although the physiological role of PSA has not yet been clarified in detail, the specific and potent inhibitors PIQ-22 and PAQ-22 (Fig. 6) showed much more potent tumor cell invasion-inhibiting activity than bestatin or actinonin (25). This suggests that PSA could be a novel target molecule for the development of antimetastatic agents. PIQ-22 and PAQ-22 are completely inactive against other aminopeptidases, including APN, which has almost the same substrate selectivity as PSA, and leucine aminopeptidase, against which bestatin and actinonin are very potent. Lineweaver-Burk plot analysis indicates that PIQ-22 and PAQ-22 are noncompetitive inhibitors of PSA, while puromycin and bestatin are competitive inhibitors (23, 25). This mode of action might explain the high specificity of PIQ-22 and PAQ-22 for PSA. Generally, aminopeptidase family members possess similar substrate selectivity, with similar structures of the substrate-binding pocket. Therefore, competitive inhibitors generally cross-inhibit aminopeptidases, as bestatin does. Because PIQ-22 and PAQ-22 are noncompetitive inhibitors, it is suggested that they bind at a specific site of PSA other than its substrate-binding site. These PSA-specific, potent, nonpeptide, small-molecule inhibitors should be useful as probes to investigate in detail the physiological function of PSA, and as lead compounds to develop superior antimetastatic agents. As an example, fluorescent probes for visualization of PSA in living cells were successfully developed (26).

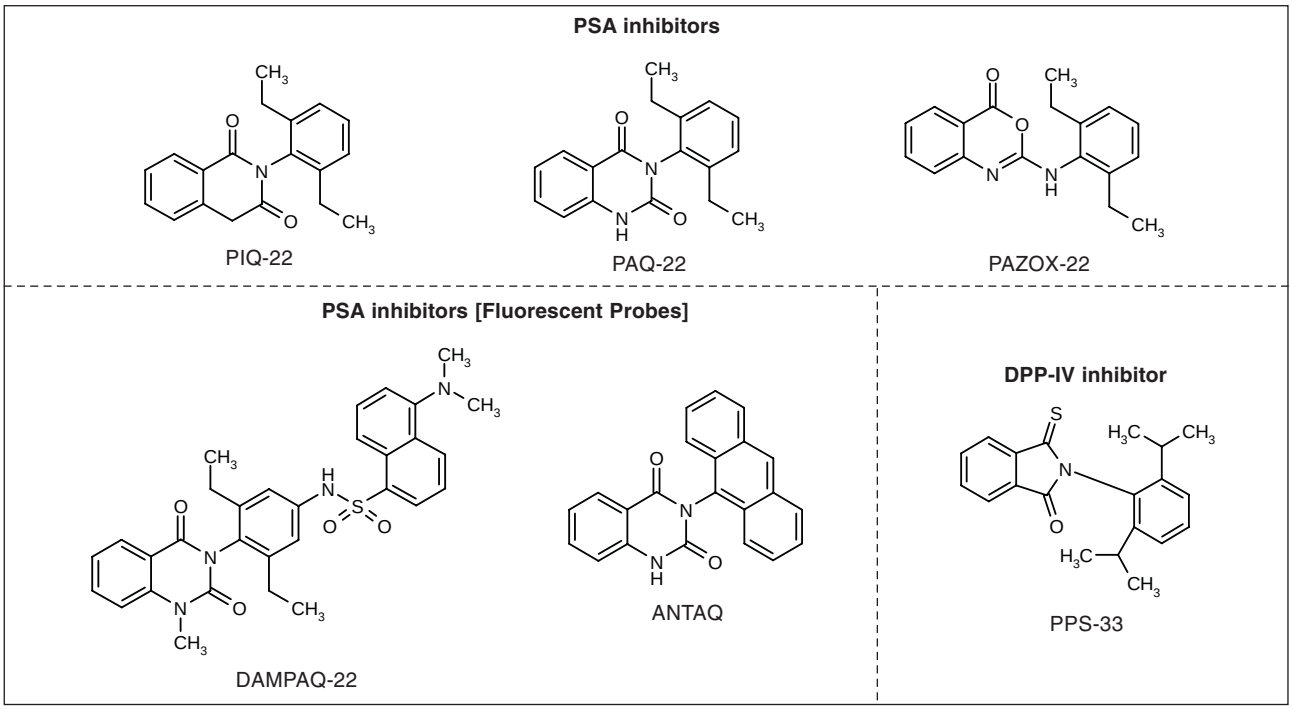


Fig. 6. Typical PSA inhibitors and a DPP-IV inhibitor.

During the structural development studies, we found some specific inhibitors of dipeptidylpeptidase IV (DPP-IV) (*e.g.*, PPS-33) (Fig. 6). DPP-IV appears to be involved in various pathophysiological effects, including tumor cell adhesion and the entry of human immunodeficiency virus (HIV) into $CD4^+$ T-cells, and therefore DPP-IV inhibitors are expected to be immunomodulators and to have potential pharmacological/clinical applications.

α-Glucosidase inhibitors

Among the pharmacological effects of thalidomide shown in Table I, only its anticachexia and antiinflammatory effects can be definitely interpreted in terms of TNF-α production-regulating activity. The antitumor-promoting effect can also be partly interpreted in terms of the same activity, but is more likely to be mainly due to antiandrogenic activity, especially in the case of prostate cancer, and COX-2-inhibiting activity, which should be related to its antiinflammatory effect. Antiangiogenic effects can be interpreted partly in terms of TNF-α production-regulating activity and partly in terms of TP/PD-ECGF-inhibiting activity. The latter activity might also play a role in thalidomide's antiviral effect. The antiviral effect, especially against HIV, might be partly explained by TNF-α production-regulating activity. The anti-cell invasion effect can be interpreted in terms of PSA-inhibiting activity.

As for the remaining hypoglycemic effect, and in part antiviral activity, we suspected that inhibition of α-glucosidase might be important. α-Glucosidase is an enzyme which catalyzes the final step in the digestion of carbohydrates. Inhibitors of this enzyme may retard the uptake of dietary carbohydrates and suppress postprandial hyperglycemia, and could be useful in the treatment of diabetes, obesity and certain forms of hyperlipoproteinemia. These inhibitors also have potential as antiviral agents controlling viral infection through interference with the normal biosynthesis of *N*-linked oligosaccharides by glycosidation of viral coat/envelope glycoproteins, and are being investigated for the treatment of both cancer and AIDS. A well-established classical α-glucosidase inhibitor is 1-deoxynojirimycin (dNM). Some derivatives of dNM have been shown to be effective against AIDS and B- and C-type viral hepatitis. Our structural development studies based on inhibiting α-glucosidase yielded potent non-competitive inhibitors (*e.g.*, CP0P) and potent competitive inhibitors (*e.g.*, CP4P) (Fig. 7) (3, 13, 27, 28). Comparison of the IC_{50} values indicates that CP0P and CP4P are about 13 and 16 times more potent than dNM, respectively.

Discussion

Our studies have indicated that the effectiveness and potential of thalidomide for the treatment of various diseases cannot be attributed solely to its TNF-α production-regulating activity. Thalidomide should be recognized as a multitarget drug. As mentioned in this article, specific and potent compounds for each of the target

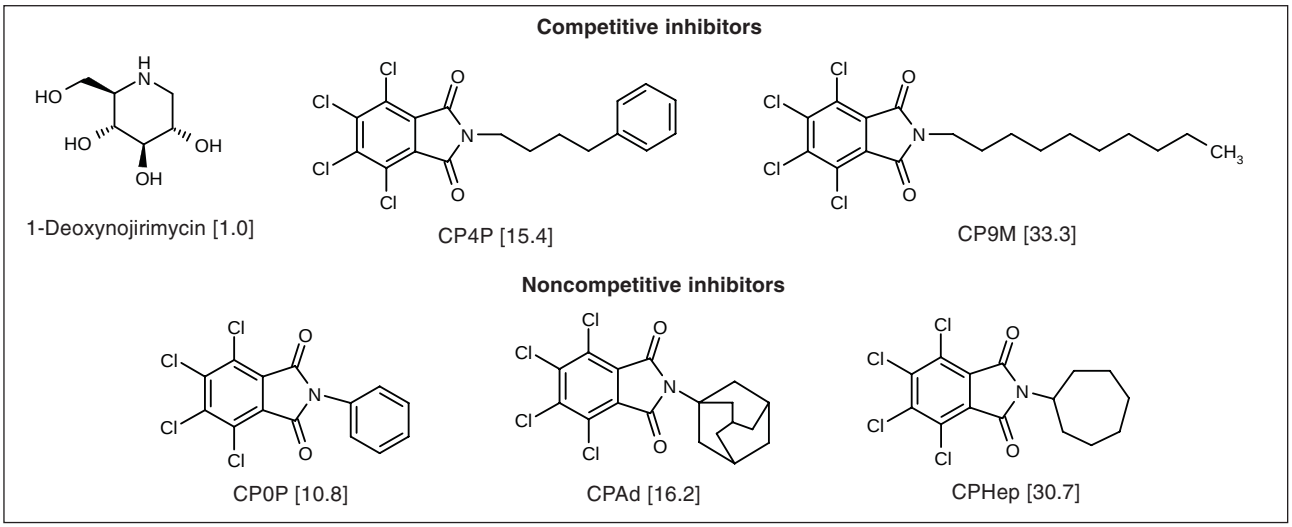


Fig. 7. Typical α-glucosidase inhibitors. Values in brackets are relative α-glucosidase-inhibiting activity compared with that of 1-deoxynojirimycin, whose activity was defined as [1.0].

molecules/phenomena listed in Table I could be prepared by appropriate modification of the thalidomide structure. This means that thalidomide intrinsically possesses pharmacophores with a wide range of activities in its small molecular skeleton. In our studies, we extracted the phthalimide and homophthalimide structures of thalidomide and by using these skeletons were able to obtain specific and potent TNF-α production regulators including bidirectional ones, pure inhibitors and pure enhancers, as well as TP/PD-ECGF inhibitors, androgen antagonists, DPP-IV inhibitors, PSA inhibitors and α-glucosidase inhibitors. We believe that the same strategy will allow the development of hypnotic, antimalarial and other agents. The creation of antiestrogens, NO synthase (NOS) inhibitors (*e.g.*, NPIQ-11) (Fig. 8) and μ-calpain inhibitors (*e.g.*, 5HPP4I) (Fig. 8) based on thalidomide structure was also partially successful. There may also be further biological effects of thalidomide other than those listed in Table I. Inhibition of phosphodiesterases and the transcription factor NF-κB are candidate actions, as well as induction of cell differentiation, which could be reproduced by phthalimide-containing retinoids (Fig. 8). Thalidomide itself has relatively low potency, or is inactive, towards some of the target molecules listed in Table I. There are at least two possible interpretations of this. One is that the overall effects of thalidomide on the target molecules are additive, and thereby appear as clinically useful effects. The other interpretation involves metabolism of thalidomide. Thalidomide is both chemically and metabolically labile, and various metabolites are known to be produced *in vivo*. Therefore, one or more metabolites might possess very potent activity on some or a specific target molecule among those listed above. In fact, teratogenicity of thalidomide has been reported to be attributed to a metabolite rather than to thalidomide itself. Also, some thalidomide metabolites are known to possess potent cell differentiation-inducing activity, which thalidomide itself does not possess.

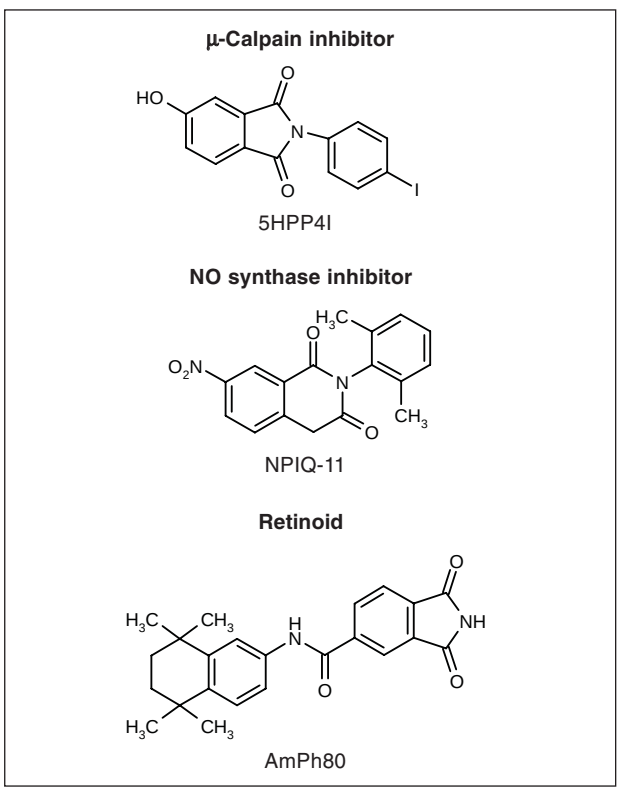


Fig. 8. Some biologically active analogues derived from thalidomide.

Finally, we should emphasize our strategy for the structural development of thalidomide. First, we identified pharmacological and biological effects of thalidomide. We

then formed a hypothesis as to the molecular target or target phenomenon which might be relevant to each pharmacological/biological effect. It is important to note that it does not matter whether thalidomide itself really binds to the hypothetical molecular target. The aim is simply to reproduce the relevant pharmacological/biological effect specifically by using newly prepared compounds. The third step is the creation of potent and specific compounds based on each biological assay system independently. Compounds thus prepared showed the corresponding single biological activity. This means that, although the compounds thus prepared all possess a phthalimide/homophthalimide skeleton as a common core skeleton, their overall structures are quite different from each other. They merely mimic thalidomide's pharmacological/biological effects, but might have no relation to thalidomide at the molecular mechanistic level. Nevertheless, we believe that by preparing compounds that mimic the pharmacological/biological effects elicited by thalidomide (even if the molecular mechanism is different from that of thalidomide), and using combinations of the prepared compounds, we will be able to reproduce or reconstruct the spectrum of pharmacological/biological effects of thalidomide.

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