Novel Indolylindazolylmaleimides as Inhibitors of Protein Kinase C-β: Synthesis, Biological Activity, and Cardiovascular Safety†

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Received June 30, 2004

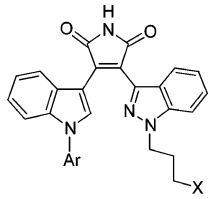
Abstract: Novel indolylindazolylmaleimides were synthesized and examined for kinase inhibition. We identified low-nanomolar inhibitors of PKC-β with good to excellent selectivity vs other PKC isozymes and GSK-3β. In a cell-based functional assay, **8f** and **8i** effectively blocked IL-8 release induced by PKC-βII (IC_{50} = 20−25 nM). In cardiovascular safety assessment, representative lead compounds bound to the hERG channel with high affinity, potently inhibited ion current in a patch-clamp experiment, and caused a dose-dependent increase of QT_c in guinea pigs.

Protein phosphorylation and dephosphorylation are important processes in the regulation of biological systems.[1] Over the past few years, considerable attention has been directed to kinases, which are responsible for phosphorylation.[2] Protein kinase C (PKC)[3] represents a family of serine/threonine kinases critically involved in signal transduction, gene expression, cell growth, and cell differentiation. From a drug discovery standpoint, the selective inhibition of individual PKC family members (isoforms) can offer a means of therapy for various human diseases.[4]

An important pharmaceutical example in the PKC area is provided by inhibitors of PKC-β, which is induced in response to hyperglycemia in cardiac, aortic, renal, and retinal tissues. Such agents could be useful in the treatment of diabetic complications, particularly retinopathy, neuropathy, nephropathy, angiopathy, macular edema, and cardiomyopathy.[5] The macrocyclic bisindolylmaleimide ruboxistaurin (LY-333531) is a potent, selective PKC-β inhibitor[6] that has advanced to phase 3 human clinical trials.[7]

During our search for PKC-β inhibitors as potential therapeutic agents, we identified novel macrocyclic bisindolylmaleimides with excellent potency (low-nM IC_{50}) against PKC-β and good selectivity over other PKC isozymes.[8] Additional kinase profiling revealed that these compounds potently inhibit (low-nM IC_{50}) glycogen synthase kinase-3β (GSK-3β).[8] Replacement of one or both indoles with 7-azaindole (or other aryl-type groups) resulted in diminished potency against PKC-β while maintaining excellent potency against GSK-3β. Thus, we obtained highly selective, potent inhibitors of GSK-3β (e.g., >300-fold selectivity over PKC-βII).[9,10] Continuing our search for potent, selective PKC-β inhibitors, we have now identified indolylindazolylmaleimides that accomplish this aim. However, in evaluating key lead compounds as preclinical development candidates, we encountered a challenge in the area of cardiovascular safety. Herein, we report on the synthesis, biological activity, and cardiovascular safety assessment for compounds in this series.

Table 1. Enzymatic Activity of *N*-(Aryl/heteroaryl)indolylindazolylmaleimides, IC_{50} (μM)[a]



compd	Ar	X	GSK-3β[b]	PKC[c] α	PKC[c] βI	PKC[c] βII	PKC[c] γ
8a[d]	vinyl	Me_2N	0.049	0.25	nd	0.023	0.32
8b	Ph	Me_2N	0.27	0.15	0.046	0.013	0.37
8c	2-Thi	Me_2N	0.37	0.22	0.015	0.010	0.28
8d	3-Py	Me_2N	0.15	0.12	0.019	0.007	0.21
8e	3-Py	no side chain[e]	0.20	1.0	0.46	0.22	nd
8f	2-Nap	Me_2N	1.9	0.057	0.033	0.005	0.11
8g	1-Nap	Me_2N	0.89	1.2	0.087	0.029	3.4
8h	4-Isoq	Me_2N	1.3	0.29	0.092	0.015	0.25
8i	3-Quin	Me_2N	3.9	0.011	0.007	0.004	0.047
8j	3-Bzthi	Me_2N	4.8	0.61	0.086	0.037	0.71
8k	2-Nap	1-Mor	2.4	0.26	0.19	0.018	0.34
8l[d]	2-Nap	1-Pyr	2.5	0.48	0.12	0.044	0.51
8m[d]	2-Nap	MePip	1.2	0.32	0.062	0.092	1.3
stau[f]				0.028	0.063	0.010	0.059

[a] Mean IC_{50} values ($n \geq 2$; see Supporting Information for error limits). nd denotes not determined. Abbreviations for Ar and X: Thi, thienyl; Py, pyridyl; Nap, naphthyl; Isoq, isoquinolinyl; Quin, quinolinyl; Bzthi, benzothienyl; Mor, morpholinyl; Pyr, pyrrolidinyl; MePip, 4-methyl-1-piperazinyl. [b] Recombinant rabbit GSK-3β was used with protein phosphatase inhibitor-2 (PPI-2) as a substrate. [c] Assay was performed as described previously (ref 6b) with the following changes: the reaction volume was reduced to 100 μL, a 96-well format was used, and [γ-^{33}P]ATP was substituted for [γ-^{32}P]ATP. [d] Prepared by a different procedure from that in Scheme 1 (see Supporting Information). [e] No aminopropyl side chain is attached to indazole nitrogen. [f] Staurosporine (ref 5b) served as a reference standard.

Target maleimides **8** (Table 1) were synthesized by standard condensation of an indole 3-glyoxylate with an indazolyl-3-acetamide (Scheme 1). Aryl and heteroaryl groups were efficiently introduced onto the indole nitrogen by treating indole (**1**) with various aryl or heteroaryl bromides in the presence of potassium carbonate and CuO to give N-arylated product **2**. The glyoxylate was introduced onto the 3-position of indole **2** by acylation with oxalyl chloride, followed by treatment with NaOMe, to afford intermediate **3**. 3-Indazoleacetic acid, **5**, was prepared by condensing 2-ni-

† This paper is dedicated to the memory of Dr. Paul A. J. Janssen, a leading figure in pharmaceutical research.

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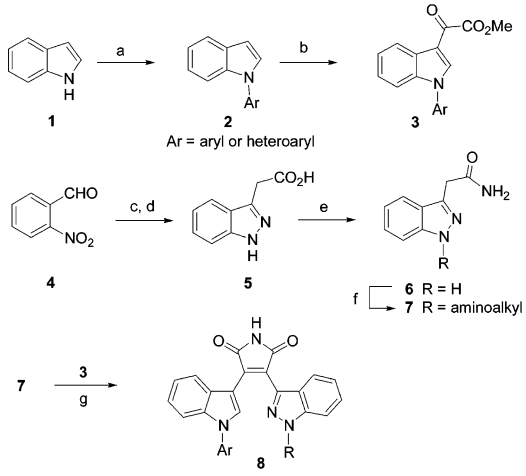
10.1021/jm049478u CCC: $30.25
Published on Web 11/13/2004

Scheme 1. Synthesis of *N*-(Aryl/heteroaryl)indolylindazolylmaleimides **8**[a]



[a] Reagents and conditions: (a) ArBr, CuO, K_2CO_3, DMF; (b) $(COCl)_2$, CH_2Cl_2, then NaOMe, 50−60% overall yield from **1**; (c) $CH_2(CO_2H)_2$, HCO_2NH_4, EtOH; (d) N_2H_4, Ra−Ni, aqueous NaOH, 54% overall yield from **4**; (e) NH_4OH, DCC, HOBt, CH_2Cl_2−DMF; (f) RBr (or RCl), NaH, DMF, 30−50% overall yield from **5**; (g) *t*-BuOK, THF, then concentrated HCl, 16−80% yield.

trobenzaldehyde with malonic acid and ammonium formate, followed by reductive cyclization under basic conditions.[11] Acid **5** was reacted with NH_4OH with the agency of dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) to give acetamide **6**, which was treated with NaH and an amine-containing alkylating agent to yield a mixture of N^1- and N^2-alkylated products in a ratio of ca. 9:1. The desired N^1-alkylated product, **7**, isolated by flash column chromatography, was condensed with glyoxylate **3** by using KO-*t*-Bu as a base,[12] followed by acidification, to furnish desired targets **8** in good to excellent yields.

The target compounds were first investigated for their inhibition of PKC-βII and GSK-3β to determine potency and selectivity, relative to our prior experience (Table 1).[8−10] Then compounds were tested for inhibition of the Ca(II)-dependent isozymes PKC-α and PKC-γ. Compound **8a** exhibited dual inhibition against PKC-βII (IC_{50} = 23 nM) and GSK-3β (IC_{50} = 49 nM),[8] with more than 10-fold selectivity over PKC-α and PKC-γ. It was possible to achieve good selectivity for PKC-βII vs GSK-3β by suitably altering the size of the group on the indole nitrogen. Thus, replacement of the *N*-vinyl in **8a** with an *N*-aryl group, such as phenyl (**8b**), thienyl (**8c**), and pyridyl (**8d**), decreased potency for GSK-3β and increased potency for PKC-βII. For example, **8c** potently inhibited PKC-βII (IC_{50} = 10 nM) and had improved selectivity (∼35-fold) vs GSK-3β; **8c** also had good selectivity vs closely related PKC-α and PKC-γ (>20-fold). The absence of the 3-dimethylaminopropyl side chain, as in **8e**, resulted in markedly reduced potency for inhibition of PKC-βII while maintaining good potency against GSK-3β. A further improvement in selectivity for PKC-βII was achieved by installing bicyclic aryl or heteroaryl groups on the indole nitrogen. The 2-naphthyl (**8f**) and 3-quinolinyl (**8i**) analogues had excellent potency for PKC-βII (IC_{50} = 4−5 nM) with ≥380-fold selectivity vs GSK-3β. Although **8f** showed moderate selectivity vs PKC-α and PKC-γ, **8i** showed little selectivity. 1-Naphthyl analogue **8g** was the most selective inhibitor of PKC-βII relative to inhibition of PKC-α (41-fold) and PKC-γ (117-fold); 3-benzothienyl analogue **8j** had the weakest potency against GSK-3β (IC_{50} = 4.8 μM). Replacement of the dimethylamino group in **8f** with other amino groups, such as morpholine (**8k**), pyrrolidine (**8l**), and *N*-methylpiperazine (**8m**), was tolerated, albeit with some loss of potency for inhibition of PKC-βII.

Table 2. Kinase Selectivity, IC_{50} (μM)[a]

kinase	8c	8d	8f	8g	8i	8j	stau[b]
PKC-δ	0.086	0.25[c]	0.49[c]	2.2[c]	0.031	1.5[c]	0.049
PKC-ϵ	0.14	0.33[c]	0.33[c]	4.2[c]	0.14	3.1[c]	0.012
PKC-ζ	57%	4.4	6.2[c]	>10	5.7	>10	>10
VEGF-R	5.0	3.7	1.0	6.9	>10	>10	0.014
CDK1	0.40	0.31	1.3	1.9	1.6	>10	0.008
EGF-R	>10	>10	>10	>10	>10	>10	0.049
PKA	6.2	>10	>10	>10	>10	>10	0.004
CK1	>10	>10	>10	>10	>10	>10	1.4
CK2	>10	>10	>10	>10	>10	>10	>10
CAMKII	7.0	3.8	6.1	>10	>10	5.6	0.006
MAPK/ERK-2	>10	>10	>10	>10	>10	>10	1.4
IRK	>10	>10	>10	>10	>10	>10	0.20
PDGF-R	2.2	5.2	5.9	>10	5.9	>10	0.002

[a] Values are the average of duplicate determinations except where indicated otherwise. For PKC-δ, PKC-ϵ, and PKC-ζ, the PKC-ϵ pseudosubstrate ERMRPRKRQGSVRRRV (16-mer peptide) was employed as a substrate. Other kinase assays were performed as described in ref 20. The % inhibition was measured at 10 μM compound. Abbreviations: VEGF-R, vascular endothelial growth factor receptor kinase; CDK, cyclin-dependent protein kinase; EGF-R, epidermal growth factor receptor kinase; PKA, cAMP-dependent kinase; CK1, casein kinase 1; CK2, casein kinase 2; CAMKII, calmodulin-dependent protein kinase II; MAPK, mitogen-activated protein kinase; ERK-2, extracellular signal-regulated kinase 2; IRK, insulin receptor kinase; PDGF-R, platelet-derived growth factor receptor kinase. [b] Staurosporine, reference standard. [c] Mean IC_{50} values (n = 2).

Representative compounds were further tested for inhibition of PKC-δ, PKC-ϵ, and PKC-ζ, as well as a panel of 10 other ATP-dependent kinases (Table 2). The compounds inhibited PKC-δ and PKC-ϵ with nanomolar to micromolar IC_{50} values and were usually highly selective over the remaining kinases except for CDK1, which was inhibited by **8c** and **8d** ($IC_{50} \approx 0.3-0.4$ μM). Compounds **8g** and **8j** are among the most selective inhibitors.

Compound **8j**, a weak inhibitor of GSK-3β and a selective inhibitor of PKC-βII vs PKC isozymes and other kinases, was docked[13] into the ATP binding sites of a homology model of PKC-βII constructed[14] on the basis of PKA (pdb code 1stc) and the crystal structure of GSK-3β (pdb code 1q3d)[15] to rationalize its selectivity. Figure 1 depicts the key interactions of this binding mode for PKC-βII, in which the maleimide of **8j** forms two hydrogen bonds with the carbonyl group of Glu-91 and the amide hydrogen of Val-93. The amine group in the long tail forms another hydrogen bond with Asp-140. These three hydrogen bonds are also formed in the GSK-3β binding model. An aromatic-stacking interaction between the benzothienyl group and Tyr-100 is unique for PKC-βII, and a pocket surrounded by the hinge loop Val-93/Asn-94/Glu-95/Gly-96/Asp-97 nicely accommodates the benzothienyl group. In GSK-3β, the corresponding Val-135/Pro-136/Glu-137/Thr-138 loop defines a smaller pocket, which is more rigid and more crowded partly due to Pro-136, such that the benzothienyl group cannot be accommodated. This difference may explain

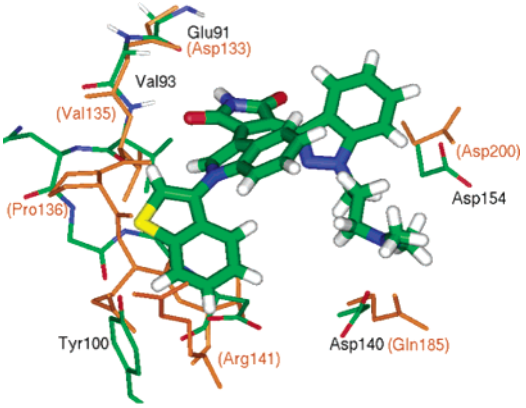


Figure 1. Docking of **8j** to PKC-βII (labeled in black) and GSK-3β (labeled in orange).

Table 3. Cellular and hERG Binding Activity[a]

compd	IL-8 release,[b] IC_{50} (μM)	hERG binding,[c] K_i (μM)
8c	0.075 ± 0.17	nd
8d	0.082 ± 0.007	0.61 ± 0.12
8f	0.024 ± 0.01	0.025 ± 0.003
8g	0.60 ± 0.22	nd
8h	0.086 ± 0.028	0.49 ± 0.06
8i	0.020 ± 0.003	0.034 ± 0.001
8j	0.63 ± 0.05	nd
8k	0.072 ± 0.038	0.85 ± 0.35
8l	0.17 ± 0.02	0.011
8m	nd	0.29 ± 0.08
stau[d]	0.077	nd
terfen[e]	nd	0.32 ± 0.04

[a] IC_{50} or K_i values are expressed as the mean ± SEM ($n \geq 2$; $n = 1$ for values without error limits). nd denotes not determined. [b] HEK293 cells stably expressing PKC-βII were preincubated with compounds prior to stimulation with PMA, and the amount of IL-8 released in cell supernatants was measured by quantitative ELISA. [c] Binding vs [^{3}H]astemizole. [d] Staurosporine, reference standard. [e] Terfenadine (ref 17), reference standard.

the excellent selectivity for PKC-βII over GSK-3β observed with **8j**.

A stable HEK293 cell line expressing PKC-βII was used to evaluate the functional activity of PKC-β inhibitors. When stimulated by the phorbol ester PMA (phorbol-12-myristate-13-acetate), these cells synthesize and release the cytokine interleukin-8 (IL-8). Selected compounds were tested in this assay to determine their ability to penetrate cell membranes and inhibit intracellular PKC-βII. As seen in Table 3, IL-8 release was blocked by several compounds, with **8f** and **8i** being very potent (IC_{50} = 20−25 nM).

In advancing the lead compounds as preclinical development candidates, we assessed their cardiovascular safety profile in vitro and in vivo. Prolongation of the QT interval of the surface electrocardiogram is associated with a predisposition for ventricular arrhythmia that may degenerate into ventricular fibrillation and sudden death.[16] Blockade of the hERG (human ether-a-go-go related gene) K^+ channel is an important molecular mechanism behind QT prolongation.[17] Since it is crucial for development candidates to be substantially free of this liability, we tested several PKC-β inhibitors for hERG binding.[18] Unfortunately, our compounds generally showed high hERG affinities (Table 3). For example, **8f**, **8i**, and **8l** had K_i values of 25, 34, and 11 nM, respectively.

Compound **8f** was evaluated further in the area of cardiovascular safety. In a patch−clamp experiment,[19]

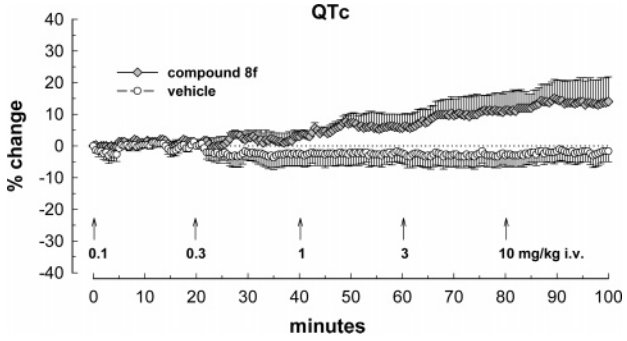


Figure 2. QT_c effects for **8f** in guinea pigs.

in which inhibition of the actual ionic current is measured, **8f** potently blocked the hERG K^+ channel with an IC_{50} of 28 nM, consistent with the hERG binding result. Intravenous infusion of **8f** into anesthetized guinea pigs caused a dose-dependent increase in the corrected QT interval (QT_c) by 7%, 11%, and 15% at 1, 3, and 10 mg/kg (Figure 2). This compound also had hemodynamic effects consisting of moderate decreases in the mean arterial pressure and heart rate at 3 and 10 mg/kg. Follow-up assessments of compounds bearing various Ar and R groups (data not shown) suggested that a basic amino group in R, which is important for achieving the high PKC-β inhibitory potency, may be a major causative factor in the undesirable cardiovascular side effects.

In summary, we have identified novel indolylindazolylmaleimides as potent inhibitors of PKC-β, some of which have excellent selectivity over PKC isozymes and GSK-3β. The best selectivity was achieved by introducing a bulky aryl/heteroaryl group on the indole nitrogen. The foremost compounds were effective intracellularly, blocking PKC-βII dependent PMA-induced IL-8 release. Cardiovascular safety studies revealed a serious potential risk with the lead compounds, involving strong binding to the hERG channel and prolongation of QT_c. Our findings with respect to cardiovascular safety not only raise concerns about compounds in our series, but also suggest a cautionary note for other PKC inhibitors of this structural class.

Acknowledgment. We thank Robert Ma for excellent technical assistance.

Supporting Information Available: Details for the synthetic procedures, characterization of products, biological assays, and sequence alignment of GSK-3β, PKA, and PKC-βII. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM049478U