Journal of Medicinal Chemistry

ARTICLE

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4-Benzimidazolyl-3-Phenylbutanoic Acids As Novel Pif-Pocket-Targeting Allosteric Inhibitors of Protein Kinase PKCζ

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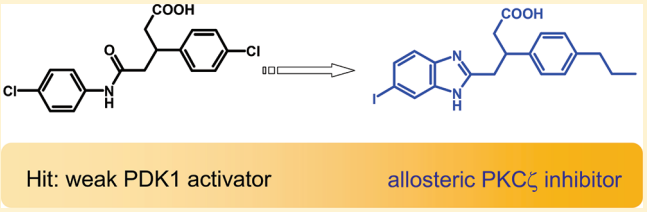
Supporting Information

ABSTRACT: Protein kinase inhibitors with an allosteric mode of action are expected to reach, in many cases, higher selectivity for the target enzyme than ATP-competitive compounds. Therefore, basic research is aiming at identifying and establishing novel sites on the catalytic domain of protein kinases which might be targeted by allosteric inhibitors. We previously published the first structure−activity relationships (SARs) for allosteric activators of protein kinase PDK1. Here, we present the design, synthesis, and SAR data on a series of novel compounds, 4-benzimidazolyl-3-phenylbutanoic acids, that inhibit the atypical protein kinace C (PKC) ζ via binding to the PIF-pocket. Key positions were identified in the compounds that can be modified to increase potency and selectivity. Some congeners showed a high selectivity toward PKCζ, lacking inhibition of the most closely related isoform, PKCι, and of further AGC kinases. Furthermore, evidence is provided that these compounds are also active toward cellular PKCζ without loss of potency compared to the cell-free assay.



INTRODUCTION

Protein kinases are an important class of drug targets and the subject of intensive pharmaceutical research. Almost all drug development programs are directed to the ATP binding pocket or sites overlapping with this pocket. However, because all protein kinases share high homology at the ATP binding site, the development of selective protein kinase inhibitors is still a challenging task. Most recently, there is increasing interest in the development of truly allosteric compounds. By interacting with an allosteric site, compounds can potentially be activators or inhibitors of protein kinase activity. However, few truly allosteric sites on protein kinases are known, and very little is known about their druggability. Moreover, the structural requirements for compounds to act either as allosteric activators or allosteric inhibitors at regulatory/allosteric sites on protein kinases have not been explored yet.

Atypical PKCs are a subfamily of PKC isoforms that are not regulated by either Ca^{2+} or diacylglycerol (DAG). Atypical PKCs (aPKCs; ζ and ι, λ is the mouse orthologue of the human ι) possess in their regulatory region an atypical C1 domain, recently found to participate in the autoinhibitory mechanism,[1] a PB1 (Phox and Bem 1) domain, which is involved in protein−protein interactions, and a pseudosubstrate domain.[2] Knockouts of PKCι/λ are embryonically lethal,[3] whereas knockouts of PKCζ result in viable mice that exhibit immunological deficiencies in the NF-κB pathway.[4,5] Therefore, PKCζ and PKCι/λ are not functionally redundant and appear to be integrated in distinct downstream signaling pathways. Using PKCζ- or PKCι-deficient mouse embryo fibroblasts, it has been shown that PKCζ rather than PKCι/λ is involved in the activation of the NF-κB pathway in this cell type via phosphorylation of Ser311 of the RelA subunit.[6,7] Recently, Levy et al. demonstrated that this phosphorylation leads to displacement of the histone methyltransferase GLP from RelA, which abrogates GLP-mediated methylation of histone H3, finally enabling NF-κB to become transcriptionally active.[8] This report established PKCζ as a critical mediator of the chromatin changes necessary for effective transcription of κB-dependent genes. Thus, there is ample evidence from knockout studies that PKCζ is required for the transcriptional activity of NF-κB in certain cell types and organs, such as, B-cells, T helper cells, T lymphocytes, the lung, and the liver.[4,5,9−11]

NF-κB plays a critical role in the control of key physiological and pathological states, from immunity and inflammation to cancer,[12] but direct inhibition by small molecules will cause toxicity because of the protective role of NF-κB in all cells and tissues.[13,14] Therefore, because of its tissue-dependent role in fine-tuning NF-κB activtity, PKCζ might be a more suitable target to pharmacologically modulate this important pathway. Because PKCζ triggers NF-κB activation and motility particularly of macrophages, PKCζ might be a pharmacological target for the treatment of inflammatory diseases such as asthma,[9]

Received: May 11, 2011
Published: August 25, 2011

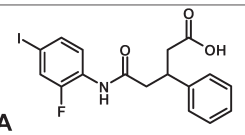
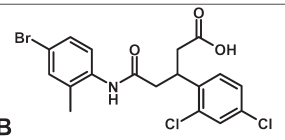
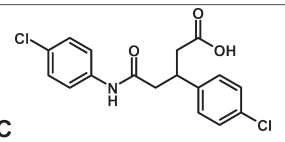
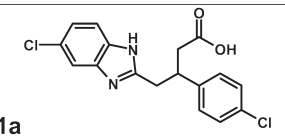
osteoarthritis,[15,16] psoriasis,[17] and sepsis.[18−20] In support of this view, TNF-induction of the expression of Il1α and Il6 was largely attenuated in PKCζ-deficient MEF cells.[8] In oncology, target validation studies have mainly been focused on PKCι so far.[21,22] However, increasing evidence points to a role of PKCζ in cell growth and survival of lymphomas and myeloid leukemias: the human lymphoma cell line U937 responds to TNFα treatment with increased PKCζ phosphorylation and activation and nuclear translocation of NF-κB, thus leading to transcription of anti-apoptotic genes.[23] Stable transfection of a kinase-dead, dominant negative PKCζ mutant in U937 cells accelerates the occurrence of apoptosis and sensitizes U937 cells grown in nude mice to etoposide.[24] In another study using the same cell line, PKCζ was also shown to confer resistance to the antileukemic drugs Ara-C and daunorubicin.[25] Similarly, PKCζ was found to mediate resistance to Fas-induced apoptosis in immature myeloid KG1a leukemic cells.[26] Furthermore, PKCζ was proposed as a new pharmacological target in follicular lymphoma, where it was identified as a direct downstream target of Rituximab. Rituximab treatment triggers the inhibition of PKCζ, which was found to promote cell survival by constitutively activating the Raf-1/mTOR pathways.[27]

A major factor limiting the pharmacological evaluation and exploitation of PKCζ as a drug target is the lack of isoform-selective inhibitors. Because of the high degree of homology in the ATP binding sites of the 11 PKC isoenzymes, rational development of ATP-competitive inhibitors selective for PKCζ seems hardly feasible; in particular, the ATP binding site of the closest homologue PKCι is lined by almost identical residues. In agreement, no PKCζ-selective inhibitors have been described in the literature up to now. Some hits from screening libraries or natural compound sources have been reported as PKCζ inhibitors, which showed some selectivity toward classical or novel PKCs, comprising hydroxyphenyl-1-benzopyran-4-ones[28] and PKCzI257.3 (*N*-(4-((dimethylamino)methyl)benzyl)-1*H*-pyrrole-2-carboxamide),[29] while no selectivity data toward the most closely related PKCι were provided. Trujillo et al. described 2-(6-phenylindazolyl)-benzimidazoles as potent PKCζ inhibitors, one of which showed some selectivity even toward PKCι (IC_{50} PKCζ = 5 nM/PKCι = 50 nM at 5 μM ATP).[30] However, that compound exhibited significant inhibition of several kinases from less related families, which is another hardly manageable feature of compounds targeting the ATP binding site.[31]

A much higher degree of selectivity can arise from targeting allosteric, regulatory sites in protein kinases. In previous studies, we and others described activators of PDK1, acting via the so-called PRK2-interacting fragment (PIF) binding pocket.[32−34] We were able to show that by binding to this site, small ligands could trigger conformational changes in the catalytic domain, thus mimicking the activatory effects of the natural peptide ligands, the phosphorylated hydrophobic motif (HM) peptides such as PIFtide.[35] PDK1 and PKC isoforms belong to the same group of AGC-Kinases; hence, there is a high degree of homology between PDK1 and PKCζ, which also possesses a PIF-pocket on the catalytic domain.

In the present study, we present the first structure−activity relationships of a novel class of allosteric PKCζ inhibitors targeting the PIF-pocket. Some reach a high degree of selectivity even toward the most closely related isoform, PKCι. In addition, the efficacy of the compounds is maintained in the cellular context.

Table 1. Inhibition of PKCζ by Hit Compounds A−C and the Newly Designed Compound 1a

	% inhibition at	
	200μM	50μM
 A	74	n.d.
 B	98	37
 C	40	0
 1a	70	0

RESULTS AND DISCUSSION

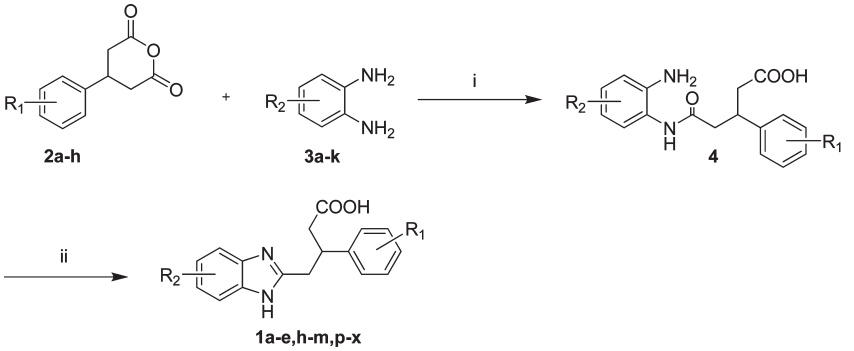
Chemistry. Our design strategy was based on compounds **A** and **B** (Table 1), which had originally been identified as weak allosteric activators of PDK1 in a previous screening campaign.[36] Further testing of these hit compounds involving related protein kinases from the AGC family revealed a weak inhibition of the atypical PKCζ as an additional biological activity. To potentially enhance the inhibitory potency of the hits, we envisaged bioisosteric replacement of the amide function involving cyclization to the benzimidazole analogues.

Key steps in the synthesis of 4-(2-benzimidazolyl)-3-phenylbutanoic acids **1** were the combination of 3-phenylglutaric anhydrides **2** with *o*-phenylenediamines **3** to form the *N*-(*o*-aminophenyl)glutaramates **4** with subsequent cyclization to the corresponding products **1** (Scheme 1).[37] The glutaramate intermediates **4** were processed further without analysis of regioisomeric composition since each pair of regioisomers would expectedly converge to a single cyclization product. Cyclization was accomplished by heating with an excess of hydrogen chloride, and the benzimidazoles were isolated as HCl salts. The only exception was derivative **1x**, which was isolated as a free base.

Except for the commercially available anhydride **2a**, all other derivatives of **2** were synthesized by a three step sequence starting from the corresponding benzaldehyde **5** (Scheme 2).[38] A piperidine-catalyzed Knoevenagel condensation−Michael addition sequence with ethyl acetoacetate led to benzalbisacetoacetates, which were isolated as the intramolecular aldol adducts **6**.[39] Double acid cleavage of compounds **6** with a concentrated sodium hydroxide solution afforded the 3-phenylglutaric acids **7**, which precipitated after acidification. Diacids **7** were converted to the 3-phenylglutaric anhydrides **2** by heating

dx.doi.org/10.1021/jm2005892 |*J. Med. Chem.* 2011, 54, 6714–6723

Scheme 1. Synthesis of 4-(2-Benzimidazolyl)-3-phenylbutanoic Acids 1[a]

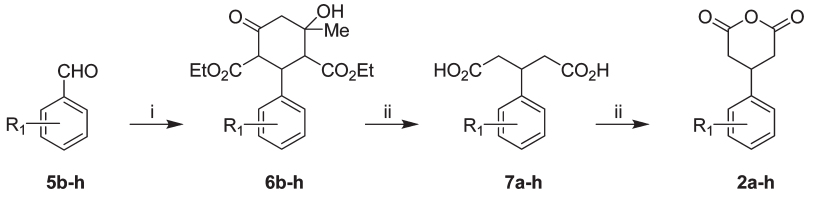


2: R_1 = 4-Cl (**a**); 3,4-Cl (**b**); 3-Cl (**c**); 4-Cl, 3-CF_3 (**d**); 4-Cl, 3-F (**e**); 2,4-Cl (**f**); 4-^{t}Bu (**g**); 4-Pr (**h**).
3: R_2 = 4-Cl (**a**); 4-F (**b**); 4-Br (**c**); 4-I (**d**); 4-CF_3 (**e**); 4-OMe (**h**); 4-COOMe (**i**); 4-CN (**j**); 4,5-F (**k**); 4-Cl, 5-F (**l**).

1: R_1, R_2	4-Cl, 5-Cl (**a**)	4-Cl, 5-F (**b**)	4-Cl, 5-Br (**c**)	4-Cl, 5-I (**d**)	4-Cl, 5-CF_3 (**e**)
yield (%)	60	60	23	78	80
1: R_1, R_2	4-Cl, 5-OMe (**h**)	4-Cl, 5-CO_2H (**i**)	4-Cl, 5-CO_2Me (**j**)	4-Cl, 5-CN (**k**)	4-Cl, 5,6-F (**l**)
yield (%)	24	74	81	82	67
1: R_1, R_2	4-Cl, 5-Cl,6-F (**m**)	3,4-Cl, 5-Cl (**p**)	3,4-Cl, 5-I (**q**)	3-Cl, 5-I (**r**)	3-CF_3,4-Cl, 5-Cl (**s**)
yield (%)	80	51	61	41	92
1: R_1, R_2	3-F,4-Cl, 5-I (**t**)	2,4-Cl, 5-Cl (**u**)	3-Cl, 5-Cl (**v**)	4-^{t}Bu, 5-I (**w**)	4-Pr, 5-I (**x**)
yield (%)	37	28	14	62	52

[a] Reagents and conditions: (i) inert solvent, RT or reflux; (ii) HCl in acetic acid or 1,4-dioxane, reflux; yields calculated over two steps.

Scheme 2. Synthesis of 3-Phenylglutaric Anhydrides 2[a]



2: R_1	4-Cl (**a**)	3,4-Cl (**b**)	3-Cl (**c**)	4-Cl,3-CF_3 (**d**)	4-Cl,3-F (**e**)	2,4-Cl (**f**)	4-^{t}Bu (**g**)	4-Pr (**h**)
yield (%)[b]	91[c]	48	38	47	51	41	28	32

[a] Reagents and conditions: (i) 2 equiv ethyl acetoacetate, cat. piperidine, EtOH, RT; (ii) 50% NaOH, reflux, then concd HCl; (iii) acetyl chloride, reflux. b: yields of **2b**–**h** were calculated over three steps based on **5**. c: yield of **2a** is based on commercial **7a**.

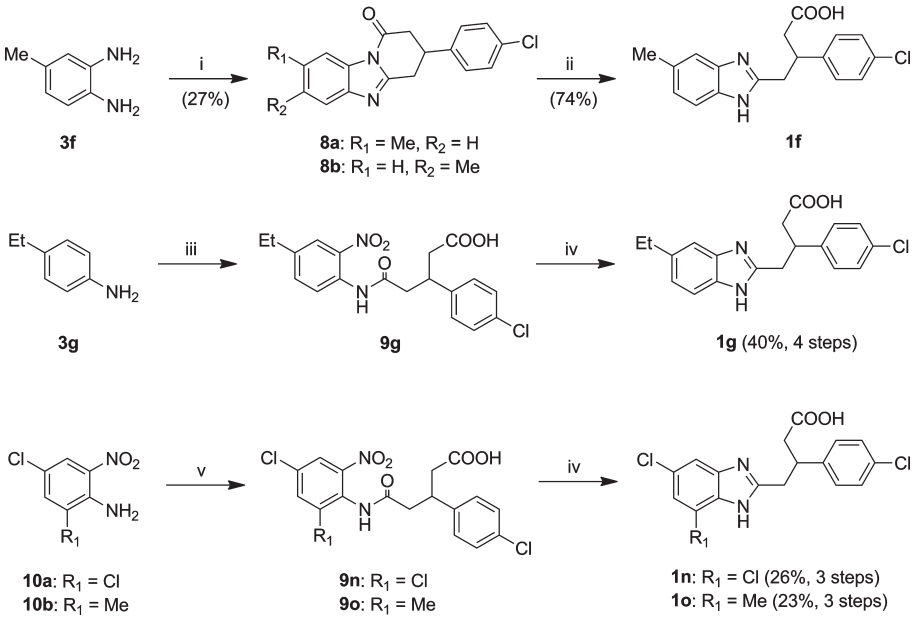
with acetyl chloride. Intermediates **6** and **7** were not further characterized.

A slightly different strategy was followed in the synthesis of benzimidazoles **1f**, **1g**, **1n**, and **1o**. Extensive reflux of a mixture of 3-(4-chlorophenyl)glutaric anhydride (**2a**) with 4-methyl-1, 2-phenylenediamine (**3f**) in an acetic acid solution without the addition of HCl provided the corresponding inner amide of the 4-(benzimidazol-2-yl)-3-phenylbutanoic acid **1f**, the 3-phenyl-3,4-dihydropyrido[1,2-*a*]benzimidazol-1(2*H*)-ones[40] **8a,b**, as a 1:1 mixture of regioisomers, in modest yield (Scheme 3). Hydrolysis of mixture **8a,b** with HCl/acetic acid led to the actual desired product **1f**. Synthesis of **1g**, **1n**, and **1o** was accomplished via the *N*-(*o*-nitrophenyl)glutaramates **9 g,n,o** which were reduced with iron powder to the corresponding *N*-(*o*-aminophenyl)-glutaramates followed by HCl promoted cyclization to the desired benzimidazoles in a one-pot procedure (Scheme 3). While *N*-(*o*-nitrophenyl)glutaramate **9g** was prepared by the nitration of *N*-(4-ethylphenyl)-3-(4-chlorophenyl)glutaramate (from anhydride **2a** and aniline **3g**), the derivatives **9n,o** were obtained by reaction of the corresponding commercial *o*-nitroanilines **10a,b** with 3-(4-chlorophenyl)glutaric anhydride (**2a**). Notably, all intermediates as well as all products appeared in a highly pure crystalline state or could be purified by recrystallization.

Structure–Activity Relationships in the Cell-Free Assay. In our initial virtual screening for PIF-binding pocket-directed compounds, conditions were chosen in a way that the identified compounds would not only specifically target the PIF-binding pocket of PDK1 but also potentially of other AGC kinases.[32,36] It was not known, however, whether binding to other AGC kinases than to PDK1 would also stabilize an active conformation of the respective kinase or rather lead to inhibition. Interestingly, when we screened some hits from our previous in silico-search[36] against a panel of AGC kinases, the 3-phenylglutaric acid monoanilides **A** and **B** were identified as weak inhibitors of PKCζ (Table 1). Our strategy to increase potency involved synthesis of

dx.doi.org/10.1021/jm2005892 |*J. Med. Chem.* 2011, 54, 6714–6723

Scheme 3. Syntheses of 4-(2-Benzimidazolyl)-3-phenylbutanoic Acids 1f,g,n,o[a]



[a] Reagents and conditions: (i) 1 equiv **2a**, THF, RT, 0.5 h; acetic acid, reflux overnight; (ii) concd HCl, acetic acid, reflux, 1.5 h; (iii) 1 equiv **2a**, 1,4-dioxane, 100 °C, 1 h; acetic acid, fuming nitric acid, RT, 1 h; (iv) 5−6 equiv iron powder, cat. HCl, acetic acid, reflux, 1 h; concd HCl, acetic acid, reflux, 2 h; (v) 1 equiv **2a**, 1,4-dioxane, RT or reflux, 1−3 h.

benzimidazoles as ring-fused analogues of the amide, also taking into account that bicyclic aryl moieties proved enthalpically favorable in our previous series of PDK1 PIF-pocket ligands.[32] First, simplified model compounds **C** and **1a** were synthesized for comparison. Benzimidazole **1a** showed an inhibitory potency similar to that of **A**, but was more potent than the open chain *p*-chlorophenyl analogue **C** (Table 1), suggesting that amide replacement by the benzimidazole moiety seemed worth while. Moreover, this result indicated that more drug-like aryl moieties such as benzoheterocycles are tolerated by subregions of the mostly hydrophobic PIF-pocket in PKCζ.

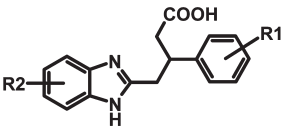
On the basis of our previous experience with the homologous PIF-binding pocket of PDK1 and considering the higher activity of **B**, we anticipated that ring substitutions with halogens or alkyl will have a large impact on potency and perhaps also on the selectivity of the inhibitors. Therefore, in order to systematically explore the ligand space available in the PIF-pocket of PKCζ, we synthesized a series of 4-benzimidazolyl-3-phenylbutanoic acid analogues (Table 2) and tested the biological activity in cell-free and cellular assays. Table 2 shows the structure−activity relationships (SARs) of this compound series. Within the series of monohalogenated benzimidazoles, 5-bromine (**1c**) increased the activity stronger than 5-chlorine (**1a**) and -fluorine (**1b**), with 5-iodine (**1d**) being most active. Interestingly, the 5-methyl (**1f**), 5-ethyl (**1g**), and 5-methoxy (**1h**) benzimidazole derivatives showed no inhibition even at 200 μM. Such a sharp contrast would not be expected if the enhancement of the hydrophobic effect alone would play a major role. While in the case of the lighter halogens, the affinity gain can partially be attributed to the -I effect, which increases the lipophilicity of the ring system, especially the iodine may be involved in halogen bonding with backbone carbonyls and/or the His289 imino nitrogen in addition. This might explain the boost of potency of the 5-iodo-benzimidazole compounds, e.g., of **1d** and **1q**. A favorable halogen bonding of iodine was also described for a class of antagonists of the HDM2-p53 interaction, which are binding to a hydrophobic groove on the surface on HDM2.[41] The 5-trifluoromethyl substitution (**1e**) gave a potency similar to the one of the 5-bromo derivative **1c**, which might also be explained by the comparable lipophilic and inductive effects on the benzimidazole ring. As for the group of compounds with more polar 5-substituents, comprising carboxyl (**1i**), carboxymethylester (**1j**), and nitrile (**1k**), the complete absence of biological activity indicates that too polar functions are not accepted by the mainly hydrophobic binding site. At any rate, these rather sharp SARs for the benzimidazole substitutions revealed the 5-/6-position as an important point for optimization of potency.

Two-fold substitution of the benzimidazole ring further increased potency, irrespective of the position, suggesting additional space surrounding this moiety. Among the 2-fold substituted benzimidazole systems, the 4,6-dichloro compound **1n** was the most potent, with an IC_{50} of 38 μM toward the purified enzyme. The 6-chloro-4-methyl derivative **1o** was less active, thus reflecting the same tendency as that observed with the monoalkyl-substituted ring.

Our previous deuterium exchange experiments with PDK1 had shown that the αC-helix lining the PIF-pocket does not exist as a stable element in solution.[35] Hence, a considerable variability of pocket size and shape was conceivable, which prompted us to introduce additional or larger substituents to probe this possibility. Indeed, introduction of a second chlorine in the *m*-position at the phenyl ring resulted in increased potency as compared to the monosubstituted counterpart (compounds **1p** vs **1a** and **1q** vs **1d** and **1r**), but 3-trifluoromethyl in **1s** could not substitute for chlorine. 3-Fluorine (compound **1t**) acted as an inert hydrogen mimick, thus representing a potential modification for improving the metabolic stability. Co-substitution at the *o*-position did not effectively increase potency (compare **1u** with **1p**) nor did

dx.doi.org/10.1021/jm2005892 |J. Med. Chem. 2011, 54, 6714–6723

Table 2. Inhibition of Recombinant PKCζ and of the NF-κB Pathway in Cells



			PKCζ inhibition [a] (cell free)			NF-κB reporter gene assay (U937 cells) [a]	
			% inhibition at [b]			% inhibition at [b]	
No.	R2	R1	200μM	50μM	IC_{50} (μM) [b]	40μM	IC_{50} (μM) [b]
1a	5-Cl	4-Cl	70	0	n.d.	11	n.d.
1b	5-F	4-Cl	30	0	n.d.	0	n.d.
1c	5-Br	4-Cl	80	25	n.d.	30	n.d.
1d	5-I	4-Cl	100	60	33	50	38
1e	5-CF_3	4-Cl	85	35	n.d.	39	n.d.
1f	5-CH_3	4-Cl	0	0	n.d.	0	n.d.
1g	5-ethyl	4-Cl	0	0	n.d.	0	n.d.
1h	5-methoxy	4-Cl	0	0	n.d.	0	n.d.
1i	5-COOH	4-Cl	0	0	n.d.	0	n.d.
1j	5-$COOCH_3$	4-Cl	0	0	n.d.	0	n.d.
1k	5-CN	4-Cl	0	0	n.d.	0	n.d.
1l	5,6-difluoro	4-Cl	50	15	n.d.	25	n.d.
1m	5-Cl, 6-F	4-Cl	85	35	n.d.	48	n.d.
1n	4,6-dichloro	4-Cl	100	60	38	80	14
1o	4-CH_3, 6-Cl	4-Cl	90	40	55	67	30
1p	5-Cl	3,4-dichloro	100	55	39	61	32
1q	5-I	3,4-dichloro	100	87	18	100	12
1r	5-I	3-Cl	70	14	n.d.	18	n.d.
1s	5-Cl	3-CF_3,4-Cl	80	20	43	57	28
1t	5-Cl	3-F,4-Cl	30	0	n.d.	0	n.d.
1u	5-Cl	2,4-dichloro	80	30	n.d.	27	n.d.
1v	5-Cl	3-Cl	0	0	n.d.	0	n.d.
1w	5-I	4-tert-butyl	80	83	23	82	13
1x	5-I	4-n-propyl	87	80	33	73	14
1y[c]	5-phenyl	4-Cl	98	90	25	75	17
1y-e1	5-phenyl	4-Cl	100	94	20	87	n.d.
1y-e2	5-phenyl	4-Cl	96	72	37	50	n.d.

[a] All compounds were tested as racemates except **1y-e1** and **1y-e2**, which are the pure enantiomers of **1y**. [b] Inhibition values are mean values of at least two experiments; standard deviation <15%; n.d., not determined. [c] Compound **1y** was synthesized as described in ref 1.

monosubstitution at the *m*-position (**1r** vs **1d**, **1v** vs **1a**), highlighting the importance of the *p*-substitution at the phenyl moiety. To further investigate the effect of larger substituents, the *p*-^{t}butyl and the *p*-^{n}propyl derivatives were synthesized.

dx.doi.org/10.1021/jm2005892 |*J. Med. Chem.* 2011, 54, 6714–6723

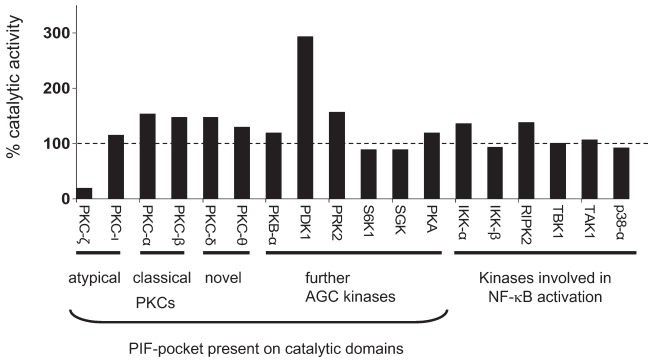


Figure 1. Selectivity profile of **1x**. Compound **1x** (50 μM) inhibits recombinant, full length PKCζ by 80%, while it does not inhibit closely related PKC isoenzymes and other AGC kinases, nor the kinases which are part of the TNFα receptor signaling complex in U937 cells (as identified by Haas et al.[44]), nor p38α MAP kinase. The catalytic activity in the presence of a 50 μM compound is given as the percentage of activity in the presence of solvent (DMSO) only, which was set 100%.

As shown in Table 2, both compounds **1w** and **1x** were inhibiting PKCζ with a potency similar to or higher than that of the *p*-Cl analogue **1d**, with **1w** almost reaching the potency of the 3,4-dichloro derivative **1q**. However, while **1q** was the most potent compound of the present series in the cell-free assay (IC_{50} = 18 μM), it lacked selectivity toward other AGC kinases (data not shown). Further analysis of selectivity among the next most potent compounds of our series revealed **1x** as the most selective congener; in particular, it did not show inhibition of the most closely related PKCι at 50 μM (Figure 1) and not even at 200 μM (not shown). While **1x** did not appreciably inhibit any of the AGC kinases tested, a weak activation (ca. 3-fold at 50 μM and 1.6-fold at 20 μM) of PDK1 was observed. The *p*-^{t}butyl derivative **1w** showed a reduced activity toward PDK1 (3-fold activation at 100 μM), while it weakly inhibited SGK (30% at 50 μM, data not shown). Altogether, the 4-position at the phenyl moiety proved very important for the enhancement of potency and fine-tuning of selectivity. A broader variation of the alkyl substituents and/or additional introduction of heterocycles might lead to compounds with a further optimized profile.

In order to test whether the bulky iodine present in the more active compounds could be replaced by a phenyl, which is comparable in size, we synthesized **1y**. This compound was found to be among the most potent PKCζ inhibitors of this study (cf., the iodine analogue **1d**, Table 2), revealing a 5-aryl moiety at the benzimidazole as another promising extension of the core structure. In addition, the selectivity of **1y** came close to that of **1x**, showing only the inhibition of S6K1 in our panel of AGC kinases (60% at 50 μM; additional data are published in ref 1).

To examine the influence of the stereochemistry on the biological activity, we aimed at separating one of the more potent racemic compounds into the enantiomers by semipreparative chiral HPLC, which was achieved in the case of **1y**. The pure enantiomers **1y-e1** and **1y-e2** both displayed activity toward PKCζ, with **1y-e1** being about 1.9-fold more potent than **1y-e2**. Thus, at least in the case of **1y**, both stereoisomers contributed significantly to the potency of the racemate. The lack of stronger chiral discrimination might be explained by the fact that the carboxylic side chain probably points to the exterior, and electrostatic interaction with Lys301 (the residue equivalent to Arg131 in PDK1) at the border of the PIF pocket might be achievable for both enantiomers via single bond rotation. In contrast, a double bond in the carboxylic side chain of our previous series of PDK1 activators strongly restricted the biological activity to the *Z* isomers only.[32]

Of note, besides **1x**, several compounds of our series which were inhibiting PKCζ also showed moderate activation of PDK1, e.g., **1m**, **1u**, and **1y** (each about 3-fold activation at 50 μM), thus corroborating the affinity for the PIF-pocket. Furthermore, compounds **1n** and **1y** completely lost the inhibitory potency toward PKCζ mutated at residues central to the PIF-pocket (PKCζ [Val297Leu] and PKCζ[Leu328Phe]; detailed descriptions of the biochemical experiments will be published elsewhere[1]). Taken together, these results suggested that the same compounds were able to target the PIF-pockets in both kinases, either causing allosteric activation or allosteric inhibition of the catalytic activity. It seems clear that the compounds do not stabilize the PIF-pocket on PKCζ in a comparable active conformation as they do on PDK1; otherwise, the enhancement of the PKCζ catalytic activity would also be expected. The PIF-pockets differ in only a few amino acids; future studies will elucidate how these differences translate into the distinct outcome with the same compounds.

Complementary to the findings reported here, we had previously identified that a PIF-pocket mutant of PDK1, PDK1-[Thr148Ala], was inhibited rather than activated by the natural ligand PIFtide.[36] Together, our data gave a first hint that the PIF-pocket on AGC kinases could also transduce the inhibition of activity by reversible ligands. In a recent report, Sadowsky and colleagues mutated the PIF-binding pocket of PDK1 introducing cysteine residues, for example, Thr148Cys, and described fragments that covalently reacted with the cysteine residues producing both activators and partial inhibitors of PDK1 activity.[42] However, it was still unknown wether reversible ligands of the PIF-pocket on an AGC kinase could trigger complete inhibition of kinase activity. The present work thus establishes the PIF-pocket of AGC kinases for the development of reversible allosteric inhibitors.

Cellular Effects on the NF-κB Signaling Pathway. Because anionic carboxylate groups can strongly impair diffusion through biological membranes, we wanted to investigate whether the compounds could exert intracellular effects on the target protein. To this end, we chose the U937 cell line as a model because of the established involvement of PKCζ in NF-κB activation.[23,24,43] Because PKCζ is strongly expressed in these cells,[43] transfection with a reporter gene plasmid expressing luciferase under control of NF-κB response elements was sufficient to monitor PKCζ-induced effects on NF-κB activation. Some initial validation experiments showed that the NF-κB activity in starved cells was strongly induced by TNFα and also weakly by C6 ceramide, a cell permeable activator lipid of PKCζ (Figure 1, Supporting Information).[23] Inhibition of this NF-κB activation after the TNFα treatment was verified by the broadband kinase inhibitor staurosporine (90% inhibition at 1 μM; Figure 1, Supporting Information). Under these assay conditions, several 4-(benzimidazol-2-yl)-3-phenylbutanoic acids were found to decrease reporter gene activity after the pretreatment of the cells (Table 2). We then compared the potency of the PKCζ inhibitors in the cellular and the cell-free activity assay. This analysis revealed a significant correlation of the decrease in luciferase activity with PKCζ inhibition in the cell-free assay (Figure 2); in particular, compounds that were inactive in the cell-free assay did not show any effect in the reporter gene assay either. Moreover, the pair of enantiomers,

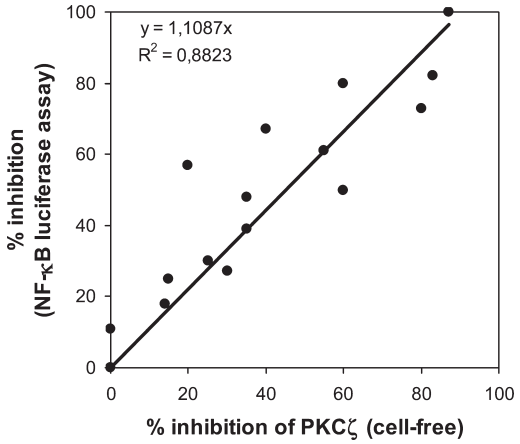


Figure 2. Strength of inhibition of recombinant PKCζ (at 50 μM) correlates with the degree of NF-κB suppression in U937 cells (at 40 μM).

1y-e1 and **1y-e2**, exhibited about the same ratio of inhibitory activity both in the cell-free and the cellular assay (Table 2).

These data suggested strongly that PKCζ is also targeted by the compounds in the cells, resulting in a disruption of the NF-κB activation cascade. In support of this notion, **1x** did not inhibit any of the other kinases associated with the TNF-Receptor 1 signaling complex (Figure 1) whose composition in U937 cells was elucidated.[44] Furthermore, the correlation between cell-free and cellular inhibition ruled out any non-specific inhibition of transcription or protein synthesis or the luciferase enzyme itself.

The most potent inhibitor in the luciferase assay like in the cell-free kinase activity assay was **1q** with IC_{50}s of 12 and 18 μM, respectively (cellular vs cell-free). As indicated by the slope of the trend line (Figure 2), no loss of potency occurred in the cell compared with that of the cell-free assay. This is a considerable advantage over ATP-competitive compounds where the high intracellular ATP concentration can cause up to a 100-fold increase of IC_{50}s.[45] Thus, our allosteric compounds displayed a remarkable cellular efficacy, although the overall affinity to the target was still rather low.

CONCLUSIONS

With the SAR study presented herein, we have provided the first evidence that it is possible to exploit the PIF-pocket on an AGC-kinase for the development of reversible allosteric inhibitors, based on the findings that (i) potency and selectivity of the leads can be markedly increased by modifying the appropriate molecule positions as identified herein and that (ii) the compounds inhibit the target kinase in cells, resulting in the effective disruption of NF-κB activation. In accordance with earlier findings on allosteric inhibitors, a remarkable degree of selectivity even toward the most closely related isoform PKCι, was observed for **1x**, thus corroborating that the optimized hit structures may represent suitable entry points for the development of isotype-specific drugs. Such compounds will not inhibit the ubiquitous components of the NF-κB activation machinery and thus will allow for more subtle and potentially more tolerable modulation of the NF-κB activity in a subset of cell types comprising the immunocompetent cells.

EXPERIMENTAL SECTION

Chemistry. Solvents and reagents were obtained from commercial suppliers and used as received. ^1H and ^{13}C NMR spectra were recorded on a Bruker DRX 500 spectrometer. Chemical shifts are referenced to the residual protonated solvent signals. The purities of the tested compounds **1a**−**y** were determined by HPLC coupled with mass spectrometry and were higher than 95% in all cases. Mass spectrometric analysis (HPLC-ESI-MS) was performed on a TSQ quantum (Thermo Electron Corporation) instrument equipped with an ESI source and a triple quadrupole mass detector (Thermo Finnigan). The MS detection was carried out at a spray voltage of 4.2 kV, a nitrogen sheath gas pressure of 4.0×10^5 Pa, an auxiliary gas pressure of 1.0×10^5 Pa, a capillary temperature of 400 °C, a capillary voltage of 35 V, and a source CID of 10 V. All samples were injected by an autosampler (Surveyor, Thermo Finnigan) with an injection volume of 10 μL. An RP C18 NUCLEODUR 100-3 (125 × 3 mm) column (Macherey-Nagel) was used as the stationary phase. The solvent system consisted of water containing 0.1% TFA (A) and 0.1% TFA in acetonitrile (B). HPLC-Method: flow rate 400 μL/min. The percentage of B started at an initial of 5%, was increased up to 100% during 16 min, kept at 100% for 2 min, and flushed back to 5% in 2 min.

3-(4-Chlorophenyl)glutaric Anhydride (2a). The suspension of commercially available 3-(4-chlorophenyl)glutaric acid (**7a**) (32.5 g, 134 mmol) in acetyl chloride (48 mL, 670 mmol) was stirred under reflux for 1 h. Then, precipitation of the product was completed by the addition of petrol ether (100 mL) and cooling to room temperature (RT). The precipitate was isolated by vacuum filtration, washed with petrol ether, and dried in vacuo to give 3-(4-chlorophenyl)glutaric anhydride (27.3 g, 91%) as colorless crystals. ^1H NMR (500 MHz, $CDCl_3$): δ (ppm) = 2.84 (dd, *J* = 17.4, 11.4 Hz, 2H), 3.09 (dd, *J* = 17.4, 4.5 Hz, 2H), 3.38−3.48 (m, 1H), 7.15 (d, *J* = 8.3 Hz, 2H), 7.37 (d, *J* = 8.4 Hz, 2H).

3-(3,4-Dichlorophenyl)glutaric Anhydride (2b). Piperidine (3 mL) was added to a solution of 3,4-dichlorobenzaldehyde (**5b**) (26.3 g, 150 mmol) and ethyl acetoacetate (39.5 g, 300 mmol) in ethanol (20 mL) at RT. The solution was kept at RT for 20 h. The voluminous precipitate was isolated by vacuum filtration and washed with ethanol until the filtrate remained colorless. After drying in vacuo, the colorless solid (the bis-adduct of ethyl acetoacetate to 3,4-dichlorobenzaldehyde **6b**) was powdered and added in portions to 50% NaOH (150 mL) with vigorous stirring. The resulting yellow slurry was stirred under reflux for 2 h. After the addition of ice water (100 mL) and cooling in an ice bath, the mixture was acidified with concd HCl to give a white precipitate. The solid was collected by vacuum filtration and washed with 1 M HCl and water. After drying in vacuo, 3-(3,4-dichlorophenyl)glutaric acid (**7b**) was obtained as a yellowish solid. The thick slurry of 3-(3,4-dichlorophenyl)glutaric acid in acetyl chloride (25 mL) was heated to reflux with stirring for 2 h. After cooling to RT, the precipitate was isolated by vacuum filtration, washed with acetyl chloride (2 × 5 mL) and petrol ether (3 × 20 mL), and dried in vacuo to give 3-(3,4-dichlorophenyl)-glutaric anhydride (**2b**) (18.8 g, 48% over three steps) as an off-white solid. ^1H NMR (500 MHz, DMSO-d_6): δ (ppm) = 2.99 (dd, *J* = 16.8, 4.9 Hz, 2H), 3.06 (dd, *J* = 16.8, 11.6 Hz, 2H), 3.58−3.65 (m, 1H), 7.31 (dd, *J* = 8.3, 2.2 Hz, 1H), 7.60 (d, *J* = 2.3 Hz, 1H), 7.63 (d, *J* = 8.3 Hz, 1H).

4-(5-Chloro-2-benzimidazolyl)-3-(4-chlorophenyl)butanoic Acid·HCl (1a). A solution of 4-chloro-1,2-phenylenediamine (**3a**) (2.85 g, 20 mmol) and 3-(4-chlorophenyl)glutaric anhydride (**2a**) (4.49 g, 20 mmol) in 1,4-dioxane (7 mL) was stirred at RT for 1 h. The precipitate was collected by vacuum filtration, washed with 1,4-dioxane, and dried in vacuo to provide a mixture of regioisomeric glutaramates as a beige colored solid. This solid was dissolved in acetic acid (10 mL) with heating. Concd HCl (4 mL) was added, and the resulting solution was heated to reflux for 2 h. Then, all volatiles were

removed at the water-jet pump, and the still hot residue was suspended in acetone (20 mL). The suspension was cooled to RT with stirring and the solid was isolated by filtration. After washings with acetone, the off-white solid was dried in vacuo to yield the hydrochloride salt of 4-(5-chloro-2-benzimidazolyl)-3-(4-chlorophenyl)butanoic acid (**1a**) (4.66 g, 60%). ^{1}H NMR (500 MHz, DMSO-d_6): δ (ppm) = 2.72 (dd, J = 16.2, 8.6 Hz, 1H), 2.83 (dd, J = 16.2, 6.2 Hz, 1H), 3.43 (dd, J = 14.9, 9.2 Hz, 1H), 3.55 (dd, J = 14.9, 6.9 Hz, 1H), 3.83−3.89 (m, 1H), 7.30 (d, J = 8.5 Hz, 2H), 7.36 (d, J = 8.5 Hz, 2H), 7.48 (dd, J = 8.8, 1.9 Hz, 1H), 7.73 (d, J = 8.8 Hz, 1H), 7.81 (d, J = 1.8 Hz, 1H). ^{13}C NMR and DEPT (125 MHz, DMSO-d_6): δ (ppm) = 32.7 (CH_2), 39.2 (CH), 39.6 (CH_2), 113.6 (CH), 115.3 (CH), 125.5 (CH), 128.3 (2 CH), 129.1 (2 CH), 129.6 (C), 130.0 (C), 131.4 (C), 131.9 (C), 140.7 (C), 153.3 (C), 172.1 (CO). MS (+ESI): m/z = 349 (M + H).

3-(4-Chlorophenyl)-4-(5-methyl-2-benzimidazolyl)butanoic Acid·HCl (1f). *7-Methyl-3-phenyl-3,4-dihydropyrido[1,2-a]benzimidazol-1(2H)-one and 8-Methyl-3-phenyl-3,4-dihydropyrido[1,2-a]benzimidazol-1(2H)-one.* The solution of 4-methyl-1,2-phenylenediamine (**3f**) (367 mg, 3 mmol) and 3-(4-chlorophenyl)glutaric anhydride (**2a**) (674 mg, 3 mmol) in THF (1 mL) was kept at RT for 0.5 h and subsequently decolorized with activated carbon. After filtration over Celite and concentration of the filtrate, the off-white residue was redissolved in acetic acid (5 mL). The solution was heated to reflux overnight. After removal of the solvent in vacuo, the residue was recrystallized from ethanol to afford a 1:1 mixture of the regioisomers 8-methyl-3-phenyl-3,4-dihydropyrido[1,2-*a*]benzimidazol-1(2*H*)-one (**8a**) and 7-methyl-3-phenyl-3,4-dihydropyrido[1,2-*a*]benzimidazol-1(2*H*)-one (**8b**) (250 mg, 27%) as colorless solid.

3-(4-Chlorophenyl)-4-(5-methyl-2-benzimidazolyl)butanoic Acid·HCl. The previous mixture (**8a**,**b**) (96 mg, 0.31 mmol) was converted to 3-(4-chlorophenyl)-4-(5-methyl-2-benzimidazolyl)butanoic acid·HCl (**1f**) (84 mg, 74%) with a mixture of acetic acid (1 mL) and concd HCl (0.5 mL) at reflux temperature for 1.5 h, with the removal of all volatiles, and trituration of the residue with acetone. ^{1}H NMR (500 MHz, DMSO-d_6): δ (ppm) = 2.43 (s, 3H), 2.72 (dd, J = 16.2, 8.6 Hz, 1H), 2.82 (dd, J = 16.2, 6.2 Hz, 1H), 3.43 (dd, J = 15.0, 9.6 Hz, 1H), 3.56 (dd, J = 15.0, 6.7 Hz, 1H), 3.83−3.91 (m, 1H), 7.26−7.31 (m, 1H), 7.29 (d, J = 8.5 Hz, 2H), 7.35 (d, J = 8.5 Hz, 2H), 7.50 (s, 1H), 7.58 (d, J = 8.4 Hz, 1H).^{13}C NMR and DEPT (125 MHz, DMSO-d_6): δ (ppm) = 20.9 (CH_3), 32.4 (CH_2), 39.2 (CH), 39.7 (CH_2), 113.1 (CH), 113.2 (CH), 126.8 (CH), 128.3 (2 CH), 128.6 (C), 129.1 (2 CH), 130.8 (C), 131.4 (C), 135.3 (C), 140.6 (C), 151.3 (C), 172.1 (CO). MS (+ESI): m/z = 329 (M + H).

3-(4-Chlorophenyl)-4-(5-ethyl-2-benzimidazolyl)butanoic Acid·HCl (1g). The solution of commercial 4-ethylaniline (**3g**) (606 mg, 5 mmol) and 3-(4-chlorophenyl)glutaric anhydride (**2a**) (1.12 g, 5 mmol) in 1,4-dioxane (3 mL) was stirred at 100 °C for 1 h. The solvent was removed, and the oily residue was redissolved in acetic acid (2 mL). With vigorous stirring at RT, fuming nitric acid (2 mL, 50 mmol) was added over the course of 1 min. After stirring at RT for 1 h, the orange solution was quenched with ice water (50 mL). The yellow gummy precipitate was separated by decantation, dried in vacuo, and redissolved in acetic acid (10 mL) and one drop of concd HCl. The solution was stirred at reflux temperature, while iron powder (1.4 g, 25 mmol) was added. After 1 h, a mixture of acetic acid (4 mL) and concd HCl (14 mL) was added with caution (foaming). Stirring at reflux was continued for 2 h to ensure the complete dissolution of the excess of iron. Then, all volatiles were removed at the water-jet pump at an elevated temperature. The residue was triturated with boiling acetone and filtered. By the addition of concd HCl, the product was precipitated from the concentrated acetone filtrate to give the hydrochloride salt of 3-(4-chlorophenyl)-4-(5-ethyl-2-benzimidazolyl)butanoic acid (**1g**) (0.75 g, 40% calc. over 4 steps) as a beige colored solid. ^{1}H NMR (500 MHz, DMSO-d_6): δ (ppm) = 1.19 (t, J = 6.3 Hz, 3H), 2.66−2.86 (m, 4H), 3.43−3.49 (m, 1H), 3.52−3.58 (m, 1H), 3.86−3.90 (m, 1H), 7.24−7.40 (m, 5H), 7.50 (s, 1H), 7.60 (d, J = 7.3 Hz, 1H), 15.2 (br s, 2H). ^{13}C NMR and DEPT (125 MHz, DMSO-d_6): δ (ppm) = 15.7 (CH_3), 28.0 (CH_2), 32.2 (CH_2), 39.0 (CH), 39.7 (CH_2), 111.8 (CH), 113.2 (CH), 125.8 (CH), 128.3 (2 CH), 128.6 (C), 129.1 (2 CH), 130.6 (C), 131.3 (C), 140.5 (C), 141.7 (C), 151.2 (C), 172.0 (CO). MS (+ESI): m/z = 343 (M + H).

Protein Kinases and Kinase Assays. PKCζ was purified as glutathione *S*-transferase (GST)-fusion protein from HEK293 cells. Cells were transiently transfected with 12.5 μg of an expression plasmid coding for full length human PKCζ (pEBG-2T-PKCζ) mixed with polyethylene imine (PEI, Polysciences Inc.) at a PEI/plasmid ratio of 10:1 (μL PEI stock solution of 1 mg/mL:μg DNA) per 14.5 cm diameter Petri dish. At 36 h post-transfection, the cells were lysed in 1.5 mL of lysis buffer (50 mM Tris-HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1% (by mass) Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 10 mM sodium β-glycerophosphate, 0.27 M sucrose, 5 mM sodium pyrophosphate, 0.1% (by volume) β-mercaptoethanol, and 1 tablet of protease inhibitor mixture (Roche) per 50 mL of buffer) per dish. The lysates were cleared by centrifugation and incubated for 2 h at 4 °C with glutathione-Sepharose. After extensive washing of the beads with lysis buffer containing 0.5 M NaCl, lysis buffer and finally in buffer A (50 mM Tris-HCl pH 7.5, 0.1 mM EGTA, 0.1% β- mercaptoethanol), the GST-fusion protein was eluted using 500 μL of buffer supplemented with 80 mM glutathione. Cell-free protein kinase activity assays were performed essentially as described previously,[36] using myelin basic protein (MBP, Millipore) as an artificial substrate (0.2 mg/mL) and started using 100 μM γ^{32}P-ATP/Mg^{2+}. Activator lipids of PKCζ such as phosphatidyl serine were omitted in order to measure true allosteric inhibition effects rather than the blocking of activation. Phosphorylated peptides were spotted on P81 phosphocellulose paper (Whatman), washed by diluted phosphoric acid, and incorporated ^{32}P quantified in a PhosphoImager. In a similar manner, PRK2 was expressed from pEBG-2T-PRK2,[46] SGK1 from pEBG-2T-SGK1-ΔN[Ser422Asp], PKBα from pEBG-2T-PKBα[Ser473Asp],[47] and PKCι from pEBG-2T-PKCι. PDK1 and S6K1 were expressed as His-tag fusion proteins in Sf9 insect cells using a baculovirus expression system from pFastBac-PDK1 and pFastBac-S6K1-T2[Thr412Glu]. PKA was purchased from Sigma; PKCα, IKKα, IKKβ, RIPK2, p38α, TAK1, and TBK1 were from Millipore; PKCβ, θ, and δ were from ProQinase. The substrates were T308tide (200 μM) for PDK1, Kemptide (100 μM) for PKA, and Crosstide (100 μM) for SGK, PKB, S6K, and PRK2. Substrates used for IKKα, IKKβ, RIPK2, p38α, TAK1, and TBK1 were described by Bain et al.[48] The activity assays for PKCα, β, θ, and δ were performed in the presence of a PKC lipid activator mix (Millipore) using 3 μM of histone H1 as the substrate.

Reporter Gene Assay. U937 cells were cultured in RPMI-1640 containing 10% FCS and penicillin/streptomycin. Transfection was performed in 6-well plates at a density of 10^6 cells/mL (2 mL per well) in the same medium without antibiotics. The transfection complex was prepared by mixing the reporter gene plasmid (pGL4.32[luc2P/NF-κB-RE/Hygro], Promega) with FuGene HD transfection reagent (Roche Diagnostics) at a ratio of 1:6 (μg DNA:μL transfection reagent) in RPMI-1640 medium according to the manufacturer's instructions. The transfection mixture was added dropwise to the wells while gently shaking, and the plates were incubated at 37 °C/5% CO_2 for 6 h. The medium was then exchanged to serum-free RPMI-1640/penicillin/streptomycin without phenol red and the cells starved overnight. The next day, test compounds dissolved in DMSO were pipetted into white 96-well plates (0.2 μL), followed by 100 μL of the transfected cells per well. After 3 h at 37 °C/5% CO_2, the NF-κB pathway was induced by the addition of 50 ng/μL of TNFα to the wells (except uninduced controls). The cells were incubated at 37 °C/5% CO_2 for 2.5 h, then the luciferase detection reagent was added (Bright-Glo, Promega, 100 μL per well)

and the luminescence measured after 5 min at RT in a plate reader (PolarStar, BMG Labtech, Freiburg, Germany).

■ ASSOCIATED CONTENT

Supporting Information. Synthetic procedures and spectroscopic data of compounds **2c**−**2h**, **1b**−**1e**, and **1h**−**1x**; separation of **1y** into its enantiomers by HPLC; validation of NF-κB reporter gene assay; and analytical HPLC chromatograph of **1y**−**e1** and **1y**−**e2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENT

We thank Professor Dr. R.W. Hartmann for generously providing lab facilities to the M.E. group and Prof. Dr. S. Zeuzem for his support of the R.M.B. lab. We acknowledge the support by the Deutsche Forschungsgemeinschaft (BI 1044/2-2) and by the Europrofession Foundation (Saarbrücken, Germany) to R.M.B. and M.E., by the Deutsche José Carreras Leukämie-Stiftung (DJCLS R 06/07) to M.E., and by the GoBio grant from the German Federal Ministry of Education and Science to R.M.B.

■ ABBREVIATIONS USED

DAG, diacylglycerol; PB1, Phox and Bem1; HM, hydrophobic motif; PIF, PRK2-interacting fragment; wt, wild type; PKCζ, protein kinase Cζ; PDK1, phosphoinositide-dependent kinase-1; GST, glutathione S-transferase

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