The compound BIRB796 inhibits the stress-activated protein kinases p38α and p38β and is undergoing clinical trials for the treatment of inflammatory diseases. Here we report that BIRB796 also inhibits the activity and the activation of SAPK3/p38γ. This occurs at higher concentrations of BIRB796 than that those that inhibit p38α and p38β and at lower concentrations than those that inhibit the activation of JNK isoforms. We also show that at these concentrations, BIRB796 blocks the stress-induced phosphorylation of the scaffold protein SAP97, further establishing that this is a physiological substrate of SAPK3/p38γ. Our results demonstrate that BIRB796, in combination with SB203580, a compound that inhibits p38α and p38β, but not the other p38 isoforms, can be used to identify physiological substrates of SAPK3/p38γ as well as those of p38α and p38β.

The stress-activated protein kinase (SAPK)1 p38 isoforms are mitogen-activated protein kinase (MAPK) family members that are activated by changes in the cellular environment, such as alterations in the concentration of nutrients, cytokines, cell-damaging agents, and changes in osmolarity of the surrounding medium (1). They comprise p38α, p38β, SAPK3/p38γ (also known as ERK6), and SAPK4/p38δ. Each p38 isoform may have different biological functions and different physiological substrates, but they all phosphorylate substrates containing the minimal consensus sequence Ser/Thr-Pro. A major challenge of current research in this field is to identify the downstream physiological substrates and processes that each p38 MAPK regulates in the cell, as well as determining which “upstream” components regulate their activities. One of the most successful aids to the identification of physiological substrates has been the use of small cell-permeable compounds that are specific inhibitors of particular protein kinases. These compounds enter cells within minutes and act rapidly to suppress the activity of a particular kinase so that indirect effects caused, for example, by changes in gene expression or protein activity, a potential risk when cells deficient in a particular kinase are used, are excluded. Moreover, the use of protein kinase inhibitors avoids the need for transfection-based approaches, which have the potential to give misleading results since the fidelity of signaling can break down when components are overexpressed.

Identification of physiological substrates for p38α and p38β has been greatly facilitated by the availability of specific inhibitors of these enzymes, such as the cell-permeant pyridinyl imidazole SB203580 and related compounds (2, 3). Substrates for p38α and p38β include other protein kinases, as well as several transcription factors and metabolic proteins (4, 5). However, little is known about the physiological substrates for SAPK3/p38γ and SAPK4/p38δ as they are not inhibited by SB203580 (6, 7), and so far there are not any commercially available inhibitors for these kinases. Nevertheless, we have recently demonstrated that the synapse-associated proteins SAP90 and SAP97 are physiological substrates of SAPK3/p38γ by using a cell-permeant peptide that blocks the interaction between SAPK3/p38γ and these PDZ domain-containing proteins (8, 9). Moreover, by using small interfering RNA technology, we have also shown that, after cellular stress, the microtubule-associated protein Tau is an in vivo substrate of SAPK4/p38δ in neuroblastoma cells (10).

Recently, a new class of p38 inhibitors has been described. These are diaryl urea compounds, which bear little structural similarity to the other class of well characterized p38 inhibitors, the pyridinyl-imidazoles. The compound BIRB796 is one of the most potent compounds of this series and binds to p38α with both slow association and dissociation rates (11). BIRB796 inhibits p38α by a novel mechanism, indirectly competing with the binding of ATP. Structure determination revealed that, prior to binding, the kinase undergoes a reorganization of the activation loop exposing a critical binding domain and yielding a structure incompatible with ATP binding (11). BIRB796 demonstrated efficacy in an endotoxin (lipopolysaccharide)-stimulated mouse model of tumor necrosis factor-α production and in a mouse model of established collagen-induced arthritis (11). BIRB796 also displayed anti-inflammatory effects in a trial of human endotoxemia and has recently been in phase III/III clinical trials for the treatment of rheumatoid arthritis (11–14).

Here we show that in addition to p38α, other MAPK family members activated by cellular stress, such as p38β, SAPK3/p38γ, and SAPK4/p38δ, are also inhibited by BIRB796. We also show that in a cell-based assay, the use of different concentrations of BIRB796, in combination with other well characterized inhibitors of p38α/β, such as SB203580, can be a useful tool for the identification of new substrates of SAPK3/p38γ and thus for the elucidation of its physiological role.

MATERIALS AND METHODS

Protein Kinase Inhibitors—The compound 1-5-tert-butyl-2-p-tolyl-2H-pyrazol-3-yl)-3-[4-(2-morpholin-4-yl-ethoxy)napthalen-1-yl]urea (BIRB796) was synthesized in four linear steps in percentage of overall
yield starting from commercially available 4,4-dimethyl-3-oxopentanenitrile following the general procedure of Regan et al. (15). SB 203580 was obtained from Calbiochem, and PD 184352 was made by custom synthesis.

Antibodies—Phospho-specific antibodies that recognize SAPK7 phosphorylated at Ser158 (antibody Phos-Ser158), Thr209 (antibody Phos-Thr209), Ser431 (antibody Phos-Ser431), or Ser442 (antibody Phos-Ser442) were raised against the peptides VSHSHpSPiK (residues 152–161), NDTSLePTTPYyNG (residues 203–215), DNHvPSYSLQGTP ASPARySjPK, and DNHvPSYSLQGTPaPSyARySPKiK (residues 427–451) of rat SAPK7 (9). An antibody that recognizes both phosphorylated and non-phosphorylated SAPK7 was generated by injecting sheep at Diagnostics Scotland (Pennicuik, UK) with glutathione S-transferase-tagged SAPK7 (9). Anti-p38β for immunoprecipitation and anti-p38α, anti-SAPK3, anti-MAPKAP-K2, anti-RSK2, and anti-ERK5 antibodies were obtained from (San Francisco, CA). Antibodies that recognize p38α phosphorylated at Thr180 and Tyr182 (these antibodies also recognize phosphorylated p38β, SAPK3/p38γ, and SAPK4/p38δ), ERK1/ERK2, phospho-ERK1/ERK2 (Thr202/Tyr204), CDK2/cyclinA, CHK1, MSK1, CSK, S6K1, aMAP-dependent protein kinase (PKA), CK1, MAPKAP-K2, serum and glucocorticoid-inducible kinase (SGK), protein kinase C-α (PKCα), and PDK1 assays; and 50 μM for the rest of the kinases listed in the table.

| Protein kinase | BIRB796 | BIRB796 |
|---------------|---------|---------|
|               | 1 μM    | 10 μM   |
| MKK1          | 71 ± 5  | 75 ± 12 |
| ERK2          | 79 ± 12 | 72 ± 3  |
| JNK1a1        | 106 ± 0 | 99 ± 1  |
| JNK2a2        | 60 ± 12 | 50 ± 8  |
| JNK3a1        | 100 ± 5 | 91 ± 6  |
| p38α          | 3 ± 1   | 3 ± 2   |
| p38β          | 7 ± 0   | 3 ± 2   |
| SAPK3/p38γ    | 29 ± 6  | 11 ± 0  |
| SAPK4/p38δ    | 47 ± 8  | 1 ± 0   |
| RSK1          | 59 ± 1  | 52 ± 8  |
| MAPKAP-K2     | 93 ± 5  | 90 ± 12 |
| MKK1          | 98 ± 2  | 83 ± 9  |
| RSK2          | 71 ± 6  | 65 ± 12 |
| PKA           | 88 ± 2  | 72 ± 2  |
| PKCa          | 80 ± 3  | 70 ± 12 |
| PDK1          | 89 ± 1  | 86 ± 0  |
| PDK2/cyclinA  | 100 ± 8 | 102 ± 5 |
| SGK           | 94 ± 13 | 93 ± 3  |
| S6K1          | 119 ± 12| 108 ± 1 |
| GSK3β         | 112 ± 5 | 107 ± 7 |
| ROCK-II       | 80 ± 3  | 78 ± 1  |
| AMPK a         | 89 ± 9  | 93 ± 2  |
| CHK1          | 96 ± 4  | 106 ± 9 |
| CK2           | 85 ± 1  | 84 ± 2  |
| PHK b         | 115 ± 0 | 102 ± 10|
| Lck           | 42 ± 5  | 18 ± 0  |
| CK1c          | 101 ± 12| 93 ± 2  |
| CDK2/cyclinA  | 102 ± 15| 93 ± 1  |
| CK1           | 87 ± 2  | 78 ± 2  |
| DYRK1α        | 82 ± 9  | 79 ± 2  |
| NER6          | 95 ± 9  | 93 ± 10 |

a AMPK, AMP-activated kinase.

b PHK, phosphorylase kinase.

c CSK, C-terminal Src kinase.

Extracts were incubated with 3 and 5 μg of the specific proteins coupled to protein G-Sepharose, respectively (Amersham Biosciences). After incubation for 2 h at 4 °C, the captured proteins were centrifuged at 13,000 g for 30 min at 4 °C, and the supernatants were removed, quick-frozen in liquid nitrogen, and stored at −20 °C until use. When required, cells were preincubated for 1 h without or with 10 μM SB 203580 or 10 μM PD 184352 or with different concentrations of BIRB796 for the times indicated in the figures.

Immunoprecipitation from Cell Lysates—MAPKAP-K2 and SAPK7 were immunoprecipitated from 0.1 to 1 mg of HEK293 cells, whereas RSK2 was immunoprecipitated from 0.1 mg of HeLa cell extract.
panel of protein kinases. We found that, at a concentration of 10 μM, BIRB796 inhibited the activity of p38α, Lck, and JNK2 as described previously (11). However, it also inhibited the activity of the other three p38 MAPK isoforms p38β, SAPK3/p38γ, and SAPK4/p38δ. It had little effect on the other protein kinases in the panel (Table I).

Time-dependent Inhibition of p38 MAPKs by BIRB796—It has been shown that BIRB796 inhibits p38α activity in a time-dependent manner due to its slow binding behavior (11). To examine whether or not BIRB796 affects the activity of other p38 isoforms similarly, we monitored the apparent inhibitory potency of the compound as a function of the time of preincubation with the kinase (Fig. 1 A). We observed firstly that BIRB796 blocked the individual p38 MAPK activities at different potencies in vitro. Thus, this compound inhibited p38α more potently than p38β and inhibited p38β more potently than SAPK3/p38γ, whereas SAPK4/p38δ was inhibited the least (Fig. 1 A and B). Secondly, the apparent IC50 value for all p38 MAPK isoforms decreased as the time of preincubation with the inhibitor increased (Fig. 1 A and B). These results are consistent with the slow binding kinetics of this compound to the p38 MAPK isoforms.

BIRB796 Is a Potent Inhibitor of p38 Isoforms in Cells—To test whether or not BIRB796 could inhibit each p38 MAPK in cells, we first investigated the effect that this compound had on the activation of one known physiological substrate of p38α, namely the protein kinase MAPKAP-K2. We incubated HEK293 cells with different concentrations of BIRB796 for 30 min or 2 h prior to stimulation with sorbitol (an osmotic shock) and examined the activation of MAPKAP-K2 by measuring its activity (Fig. 2 A). MAPKAP-K2 activation was inhibited in these cells in a time-dependent manner with an apparent IC50 of 30 nM after 30 min or 8 nM after 2 h of preincubation with BIRB796 (Fig. 2 A). MAPKAP-K2 activation was also blocked by preincubation of the cells with the p38 MAPK inhibitor SB203580 as described previously (2) (Fig. 2 A).

We also examined the phosphorylation of p38α and p38β under the same conditions and observed that the phosphorylation of these two kinases was also blocked by pretreatment of the cells with the compound (Fig. 2 B and C). These results suggest that the binding of BIRB796 to these p38 MAPKs is also impairing their phosphorylation by the upstream kinases MKK6/MKK3 and/or enhancing their dephosphorylation. On the other hand, preincubation of cells with the SB203580 did not prevent p38α phosphorylation by this agonist (Fig. 2 B), although it blocked p38β phosphorylation, as shown previously (5) (Fig. 2 C).

To test whether BIRB796 could also inhibit the phosphoryl-
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Fig. 2. Effect of BIRB796 on the activity and activation of p38α and p38β in cells. A, BIRB796 inhibits the activation of MAPKAP-K2. HEK293 cells were preincubated for 2 h (gray bars) or 30 min (black bars) in the presence of the indicated concentrations of BIRB796 or preincubated for 1 h in the presence of 10 μM SB203580. Cells were stimulated for 30 min with 0.5 M sorbitol in the continued presence or absence of the inhibitors and lysed. MAPKAP-K2 was immunoprecipitated from 0.2 mg of lysate protein and assayed as described under "Materials and Methods." B, BIRB796 inhibits the activation of p38α. Lysates (50 μg) from HEK293 treated as in panel A were immunoblotted using either an antibody that recognizes phosphorylated p38α (Phos-p38α) or an antibody that recognizes both phosphorylated and unphosphorylated p38α. C, BIRB796 inhibits the activation of p38β. C2C12 myoblasts were treated as in panel A, and p38β was immunoprecipitated from 3 mg of cell lysate and immunoblotted with either the p38α phospho-specific antibody, which also recognizes p38β, or an antibody that recognizes both phosphorylated and unphosphorylated p38β.

Fig. 3. Effect of BIRB796 on the activation of SAPK3/p38γ. A, HEK293 cells were preincubated with different concentrations of BIRB796 or 10 μM SB203580 as indicated in Fig. 2A before being exposed to 0.5 M sorbitol for 30 min. Cell lysates (50 μg) were immunoblotted with either the p38α phospho-specific antibody (Phos-p38α), which also recognizes phosphorylated SAPK3/p38γ (Phos-SAPK3), or an antibody that recognizes both phosphorylated and unphosphorylated SAPK3/p38γ (SAPK3). B, the intensity of the bands in the immunoblots from panel A was quantified as described under "Materials and Methods," and the percentage of inhibition of SAPK3/p38γ phosphorylation was calculated. Results in panel B are shown as the mean ± S.E. for duplicate determinations from two experiments.

Inhibition of SAPK3/p38γ and SAPK4/p38γ, cells were preincubated with different concentrations of BIRB796 for 30 min or 2 h before stimulation with sorbitol. The phosphorylation of SAPK3/p38γ was blocked by this compound (Fig. 3A) with an apparent IC₅₀ of 90 nM or 35 nM after 30 min or 2 h preincubation, respectively (Fig. 3B), which are 3 and...
4-fold higher than the IC_{50} for blockade of MAPKAP-K2 activation. Since the expression level of SAPK4/p38γ in HEK293 cells is very low and this isoform is not activated in these cells after sorbitol treatment (9), the effect of BIRB796 on the activation of this kinase was studied in mouse embryonic fibroblasts. Surprisingly, SAPK4/p38γ phosphorylation after osmotic shock was enhanced at low doses of the inhibitor and blocked at higher concentrations of BIRB796 (Fig. 4). The apparent IC_{50} for SAPK4/p38γ was ∼20-fold higher than the IC_{50} for SAPK3/p38γ. Phosphorylation of SAPK4/p38γ after stimulation was also enhanced when cells were preincubated with the p38α/p38β inhibitor SB203580 (Fig. 4). One possible explanation for this result is that p38α/p38β negatively regulates SAPK4/p38γ activation by an unknown mechanism. Alternatively, the binding of BIRB796 and SB203580 to p38α/p38β may allow MKK6 and/or MKK3 to activate SAPK4/p38γ preferentially.

**BIRB796 Blocks the Phosphorylation of the SAPK3/p38γ Substrate SAP97 in Cells**—The scaffold protein SAP97 is the mammalian homologue of the *Drosophila* discs large tumor suppressor and a physiological substrate of SAPK3/p38γ. SAP97 becomes phosphorylated in HEK293 cells at four major residues (Ser^{158}, Thr^{209}, Ser^{431}, and Ser^{442}) in response to osmotic stress (9) (Fig. 5A). The phosphorylation of all four residues was greatly reduced when cells were pretreated with 1 μM BIRB796 before stimulation but not by pretreatment with 10 μM SB203580 (Fig. 5A). The apparent IC_{50} for inhibition of Ser^{158} phosphorylation was 150 nM or 60 nM after 30 min or 2 h preincubation, respectively (Fig. 5, B and C), similar to the IC_{50} for inhibition of SAPK3/p38γ.

**BIRB796 Inhibits JNK Activation in Vivo**—We also examined the effect of the inhibitor BIRB796 on the activation of the c-Jun N-terminal kinase (JNK), which is also a MAPK family member activated by stress. Exposure of cells to osmotic shock caused an increase in the phosphorylation of both alternative spliced isoforms (46 and 54 kDa) of JNK1 and JNK2, which was completely impaired when cells were preincubated with 10 μM BIRB796 (Fig. 6A). Inhibition of p46(JNK1/2) phosphorylation was more sensitive to BIRB796 than p46(JNK1/2) phosphorylation (Fig. 6A). Thus, after a 2-h preincubution with the inhibitor, the apparent IC_{50} for p46(JNK1/2) was 1 μM, and for p54(JNK1/2), it was 350 nM, whereas the apparent IC_{50} for p54(JNK1/2) was higher than 1 μM when cells were incubated for 30 min with BIRB796 prior to stimulation. These results suggest that BIRB796 inhibits JNK by a mechanism similar to the p38 MAPK isoforms.

The phosphorylation of one physiological substrate of JNK, the transcription factor c-Jun, was also examined (Fig. 6B). After exposure of the cells to osmotic shock in the presence of increasing concentrations of BIRB796, c-Jun was immunoblotted using a phospho-specific antibody, which specifically recognizes Ser^{63}. Phosphorylation of c-Jun was completely blocked at 10 μM BIRB796 with an apparent IC_{50} of 1 μM after 2 h of preincubation with the inhibitor (Fig. 6B), similar to the IC_{50} for inhibition of p46(JNK1/2) activation under these conditions (Fig. 6A). Taken together, these results show that BIRB796 blocks JNK1/2 activation and activity in HEK293 cells but at higher concentrations than those needed to block p38α, p38β, or SAPK3/p38γ.

**BIRB796 Does Not Inhibit ERK1/2 or ERK5 Activation in Vivo**—To test further the specificity of BIRB796, we examined its effect on the activation and activity of other MAPK family members, ERK1/2 and ERK5. These experiments were carried out in HeLa cells in which the activation of the ERK1/2 and ERK5 pathways by EGF is well characterized (21).

Phosphorylation of ERK1/ERK2 and activation of RSK2, one of their in vivo substrates, were not prevented by preincubation for up to 2 h of the cells with even 10 μM BIRB796, whereas preincubation with the classical MAPK pathway inhibitor PD184352 completely blocked ERK1/ERK2 phosphorylation.
and RSK2 activation (Fig. 7, A and B). These results indicated that BIRB796 does not inhibit the activation and activity of ERK1/ERK2.

The phosphorylation of ERK5 could be detected by a small decrease in its electrophoretic mobility using an antibody that recognizes the phosphorylated and dephosphorylated forms of the protein equally well (21). Preincubation of HeLa cells with PD184352 inhibited the EGF-induced phosphorylation of ERK5, as expected from earlier studies (21, 22) (Fig. 7C). However, increasing concentrations of BIRB796 did not affect the mobility of the kinase caused by its phosphorylation and therefore did not block the activation of ERK5 induced by EGF (Fig. 7C).

**DISCUSSION**

Recently, it has been described that the diaryl urea compound BIRB796 is a highly selective inhibitor of the protein kinase p38α (11). To establish the specificity of a particular inhibitor is a critical issue as a means for therapeutic intervention. Since protein kinases are a large class of enzymes, most of which belong to the same family, and the degree of homology across the entire family is relatively high, especially within the catalytic core (23), it is difficult to develop compounds that inhibit one particular protein kinase without inhibiting several related enzymes. For this reason, we decided to reexamine the specificity of the BIRB796 compound. The work described in...
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The present study shows that BIRB796 inhibits the activity in vitro, and the activation in the cell, of all p38 MAPK and JNK1/2 isoforms.

Structural analysis of p38α has shown that BIRB796 binds to a novel site within the ATP-binding pocket of the kinase, which is created by a conformational change in the enzyme induced by the inhibitor, which yields to a structure incompatible with ATP binding (11). Moreover, solution studies have demonstrated that this class of compound has slow binding kinetics, consistent with the requirement for a conformational change (11). Our in vitro and in vivo data show that BIRB796 inhibits all p38 MAPK and JNK isoforms in a time-dependent manner, suggesting that the association of this compound with each of these kinases is slow, probably due to the requirement of the previously described conformational change. BIRB796 binding is targeted to the Phe residue in the conserved DFG motif that is buried in a hydrophobic pocket between the two major lobes of the kinase domain (11). Although the residues in this pocket are highly conserved among all of these kinases, we have observed that BIRB796 is more selective for p38α and p38β than for the SAPK3/p38γ and SAPK4/p38δ isoforms and other members of the MAPK family. This could be due to the fact that this diaryl urea inhibitor also utilizes the hydrophobic pocket containing Thr106 (11) a residue unique to p38α and p38β.

Moreover, we also show that BIRB796 impairs the phosphorylation of p38 MAPKs or JNKs by the upstream kinase MKK6 or MKK4 but does not affect their dephosphorylation in vivo. Our results suggest that the conformational change caused by the binding of the inhibitor to the MAPK may affect the structure of both its phosphorylation site and the docking site for the upstream activator, therefore impairing the phosphorylation of p38 MAPKs or JNKs.

We also show that incubation of cells with BIRB796 prior to stimulation blocks phosphorylation of the physiological substrates of p38α and SAPK3/p38γ. In particular, BIRB796 impairs the phosphorylation induced by osmotic shock of SAP97, which is a SAPK3/p38γ substrate.

Recently, we have shown that the phosphorylation of different PDZ domain-containing proteins by SAPK3/p38γ, such as SAP97, is dependent on the interaction of the C-terminal se-
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The results presented in this study show that it is possible to vary the concentration of BIRB796 in the culture medium to differentially inhibit particular stress-activated protein kinases in combination with the more specific p38α/β inhibitor SB203580. For example, in HEK293 cells exposed to osmotic shock that activates p38 MAPKs and JNKs, BIRB796 at 0.1 μM inhibits p38α/β specifically, whereas at 0.3 μM, it also inhibits SAPK3/p38γ. Thus, physiological substrates for SAPK3/p38γ can be identified by identifying proteins, the phosphorylation of which is completely blocked by preincubation of HEK293 cells with 0.3 μM BIRB796 but not by 10 μM SB203580. At higher concentrations than 1 μM, BIRB796 also substantially blocks the JNK pathway. However, the precise concentration needed for inhibition may vary from cell to cell. For this reason, it is important to examine the minimum concentration of BIRB796 required to suppress the activity of a particular MAPK by 80–90% by checking in parallel the phosphorylation of a validated substrate of the protein kinase studied.

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BIRB796 Inhibits All p38 MAPK Isoforms in Vitro and in Vivo
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