Conversion of SB 203580-insensitive MAP kinase family members to drug-sensitive forms by a single amino-acid substitution

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Background: Specific inhibitors of protein kinases have great therapeutic potential, but the molecular basis underlying their specificity is only poorly understood. We have investigated the drug SB 203580 which belongs to a class of pyridinyl imidazoles that inhibits the stress-activated protein (SAP) kinases SAPK2a/p38 and SAPK2b/p38β2 but not other mitogen-activated protein kinase family members. Like inhibitors of other protein kinases, SB 203580 binds in the ATP-binding pocket of SAPK2a/p38.

Results: The SAP kinases SAPK1γ/JNK1, SAPK3 and SAPK4 are not inhibited by SB 203580, because they have methionine in the position equivalent to Thr106 in the ATP-binding region of SAPK2a/p38 and SAPK2b/p38β2. Using site-directed mutagenesis of five SAP kinases and the type I and type II TGFβ receptors, we have established that for a protein kinase to be inhibited by SB 203580, the sidechain of this residue must be no larger than that of threonine. Sensitivity to inhibition by SB 203580 is greatly enhanced when the sidechain is even smaller, as in serine, alanine or glycine. Thus, the type I TGFβ receptor, which has serine at the position equivalent to Thr106 of SAPK2a/p38 and SAPK2b/p38β2, is inhibited by SB 203580.

Conclusions: These findings explain how drugs that target the ATP-binding site can inhibit protein kinases specifically, and show that the presence of threonine or a smaller amino acid at the position equivalent to Thr106 of SAPK2a/p38 and SAPK2b/p38β2 is diagnostic of whether a protein kinase is sensitive to the pyridinyl imidazole class of inhibitor.

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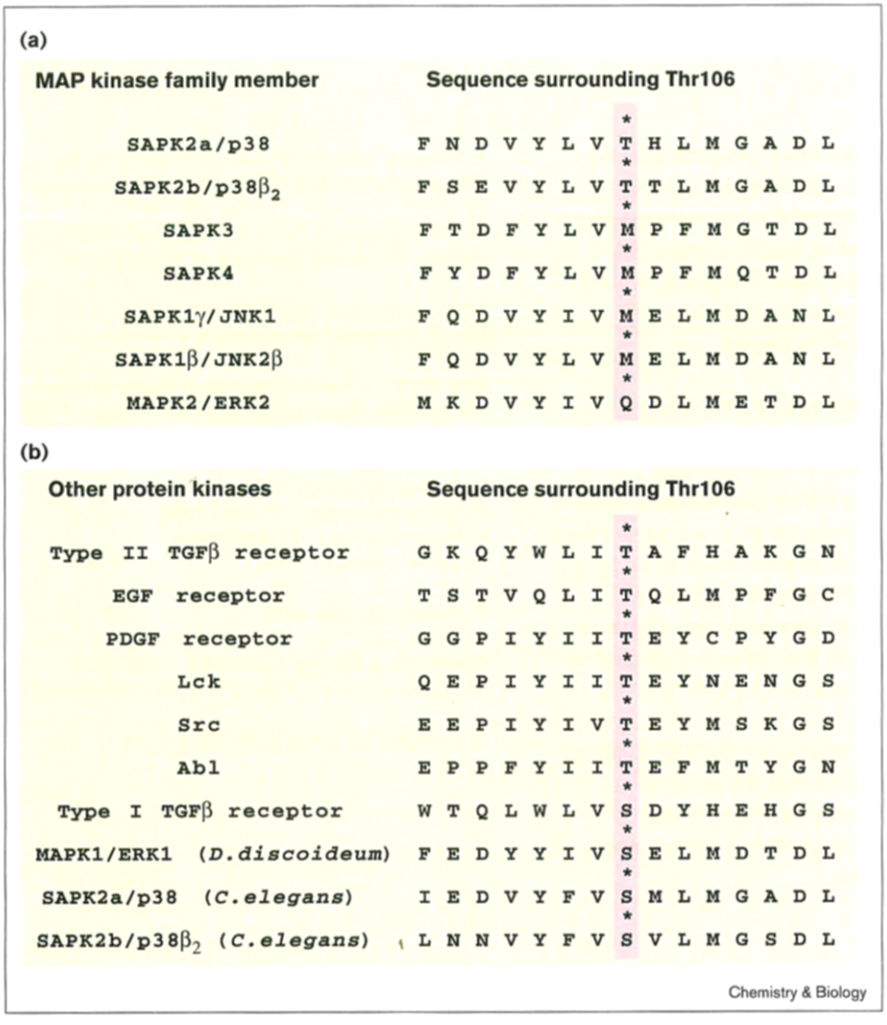
Introduction

Protein kinases form one of the largest families of proteins encoded in the human genome, and these enzymes have pivotal roles in almost all aspects of cell regulation. Abnormal protein phosphorylation is the cause or consequence of many diseases and, for this reason, protein kinases have become attractive targets for drug therapy. Several relatively specific inhibitors of these enzymes have been developed that have therapeutic potential for the treatment of cancer, diabetes, hypertension and inflammation [1–3]. A class of pyridinyl imidazoles suppresses the synthesis (and some of the actions) of pro-inflammatory cytokines, and shows promise for the treatment of rheumatoid arthritis and other chronic inflammatory conditions [4]. These pyridinyl imidazoles are remarkably specific inhibitors of stress-activated protein (SAP) kinase 2a (SAPK2a, also termed p38, RK and CSBP2) and SAP kinase 2b (SAPK2b, also termed p38β2) [5,6] and have been used to identify physiological substrates of these protein kinases, such as the protein kinases MAPKAP kinases 2/3 and Mnk1/2, and several transcription factors (reviewed in [7]).

SAPK2a/p38 and SAPK2b/p38β2, which share 74% sequence identity, are members of the mitogen-activated protein (MAP) kinase family [6,8–11]. Other members of this family, however, whose amino-acid sequences are 40–60% identical to SAPK2a/p38 and SAPK2b/p38β2 are not inhibited by the pyridinyl imidazoles SB 203580 or SB 202190. These enzymes include SAPK1 (or JNK), of which there are a number of closely related isoforms that phosphorylate the transactivation domain of c-Jun [5,12,13] and the more recently identified SAPK3 (also called ERK6 and p38γ) [14–17] and SAPK4 (also called p38δ) [6,10,18,19] whose physiological substrates are unknown.

SB 203580 binds SAPK2a/p38 competitively with ATP [20], and the determination of the three-dimensional structure of SAPK2a/p38 in a complex with a closely related pyridinyl imidazole has established that these drugs insert into the ATP-binding pocket of SAPK2a/p38 [21,22]. These analyses also revealed that the 4-fluorophenyl ring of the drug does not make contact with residues in the ATP-binding pocket that interact directly with ATP. One residue near the 4-fluorophenyl ring is Thr106 and mutation of this residue to methionine makes SAPK2a/p38 insensitive to the drug [22]. Thr106 is conserved in SAPK2b/p38β2, but is replaced by methionine in SAPK1γ/JNK1, SAPK3 and SAPK4 (Figure 1). We therefore examined the role played

Figure 1



Amino-acid sequences surrounding Thr106 in SAPK2a/p38 and SAPK2b/p38β2 (the residue that confers sensitivity to SB 203580). **(a)** Human MAP kinase family members. **(b)** Protein kinases with threonine or serine in the position equivalent to Thr106 of human SAPK2a/p38. The residues in the position equivalent to Thr106 in human SAPK2a/p38 are marked by an asterisk.

by the threonine/methionine residue in conferring sensitivity or resistance to the pyridinyl imidazole drugs on these and other protein kinases. SAPK1/JNK1, SAPK3 and SAPK4 became sensitive to SB 203580 following mutation of this methionine to threonine or a smaller amino acid. The presence of serine or threonine at this position was also found to be diagnostic of whether more distantly related protein kinases are sensitive to inhibition by SB 203580.

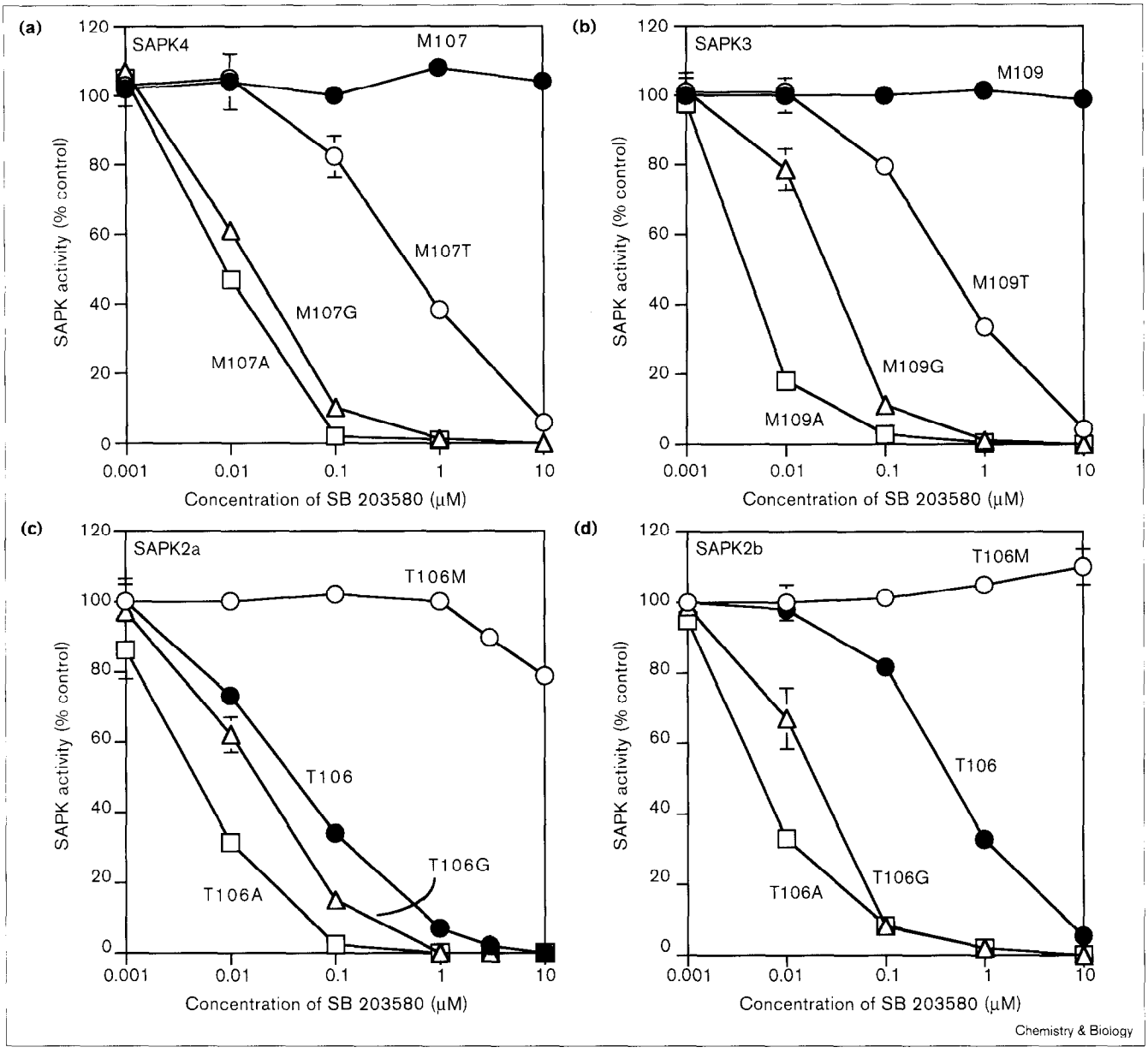
Results

Inhibition of SAP kinase mutants by SB 203580

SAPK3 and SAPK4 both became sensitive to SB 203580 when methionine in the equivalent position of Thr106 in SAPK2a/p38 was changed to threonine (Figure 2). Further mutagenesis to a variety of other residues revealed that a sidechain smaller than that of threonine, as in serine, alanine or glycine, made SAPK3 more sensitive to the drug (IC_{50} = 10–50 nM), whereas inhibition was extremely poor when a large residue, such as glutamine, was present at this position (Table 1). Glutamine is found at this position in MAP kinase family members MAPK1/ERK1 and MAPK2/ERK2. The Met109→Ala and Met109→Gly mutants of SAPK3 had the same K_{cat}/K_m values as the wild-type enzyme (data not shown).

SAPK4, SAPK2a/p38 and SAPK2b/p38β2 also became more sensitive to SB 203580 when glycine or alanine was present at this position (IC_{50} = 15–30 nM; Figure 2). Human wild-type SAPK2a/p38 was inhibited about tenfold more potently than human wild-type SAPK2b/p38β2 or the threonine mutants of SAPK3 and SAPK4 (Figure 2). The sensitivities of all these enzymes to SB 203580 became similar, however, after mutation of Thr106 to glycine or alanine (Figure 2). The alanine mutants were inhibited more strongly than the glycine mutants, suggesting that the size of the alanine sidechain is optimal for inhibition by SB 203580. Even though SAPK2a/p38 and SAPK2b/p38β2 are less similar to the

Figure 2



Inhibition of wild-type and mutant SAPK2a/p38, SAPK2b/p38β2, SAPK3 and SAPK4 by SB 203580. The effect of the drug on the wild-type enzymes is shown by the closed circles and its effect on mutant enzymes by open symbols. Mutations are designated using single-letter amino-acid code (for example, M107T, Met107→Thr). **(a, b)** Wild-type SAPK4 and SAPK3 are resistant to SB 203580, because these kinases have methionine residues at positions 107 and 109, respectively. **(c)** Wild-type SAPK2a/p38 has a threonine residue at position 106 and is inhibited by SB 203580 with an IC_{50} value of 0.08 μM. Thr106→Met SAPK2a/p38 is still inhibited by SB 203580, but the IC_{50} value has increased to 100 μM. **(d)** Wild-type SAPK2b/p38β2 has a threonine residue at position 106 and is inhibited by SB 203580, with an IC_{50} value of 1 μM. Thr106→Met SAPK2b/p38β2 is resistant to SB 203580.

isoforms of SAPK1/JNK (40% identity) than to SAPK3 or SAPK4 (60% identity) [8–19], SAPK1γ/JNK1 became sensitive to inhibition by SB 203580 when Met108 was mutated to threonine (Figure 3). The IC_{50} value (10 μM) for the Met108→Thr SAPK1γ kinase was 30- to 50-fold higher than for SAPK2b/p38β2 or the threonine mutants of SAPK3 or SAPK4, and 500-fold higher than for wild-type SAPK2a/p38 (Figure 2). After mutation to alanine, however, SAPK1γ/JNK1 was inhibited by SB 203580 at submicromolar concentrations (Figure 3). SAPK1γ/JNK1 differs from other SAP kinases by the presence of isoleucine at position 106 instead of leucine [13]. When residue 106 was changed to leucine in the wild-type enzyme, SAPK1γ/JNK1 was inhibited to some extent by

Table 1

Effect of mutating Met109 on the sensitivity of SAPK3 to SB 203580.

Residue at position 109	IC_{50} value (μM)
Methionine	>100
Phenylalanine	>100
Lysine	55
Glutamine	50
Leucine	45
Glutamate	45
Threonine	0.3
Serine	0.05
Glycine	0.03
Alanine	0.01

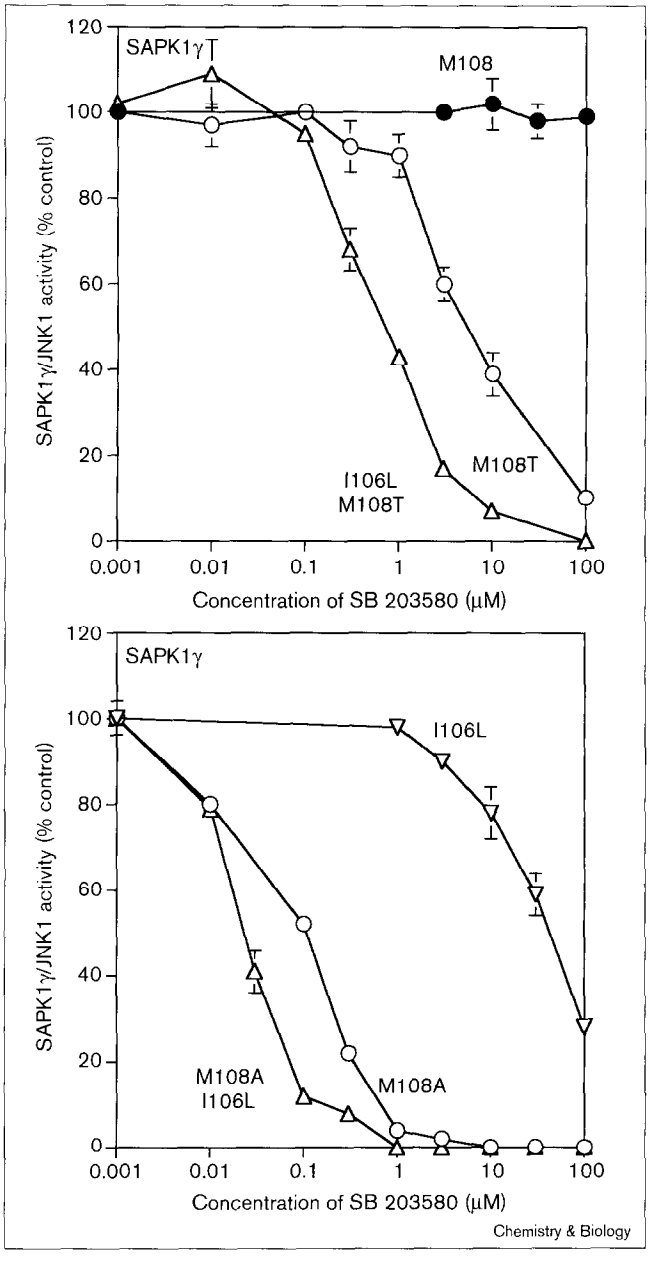
Site-directed mutagenesis was used to change residue 109 in SAPK3 from methionine to one of nine other amino acids.

SB 203580, with an IC_{50} value of 50 μM (Figure 3). When Ile106 was changed to leucine in the Met108→Thr and Met108→Ala mutants, SAPK1γ/JNK1 was strongly inhibited by SB 203580, with an IC_{50} value of 30 nM for the Ile106→Leu Met108→Ala SAPK1γ/JNK1 (Figure 3). These results demonstrate that residue 108 is the major determinant for inhibition of SAPK1γ/JNK1 by pyridinyl imidazoles and that residue 106 plays a minor role.

Inhibition of Lck and the type II TGFβ receptor by SB 203580

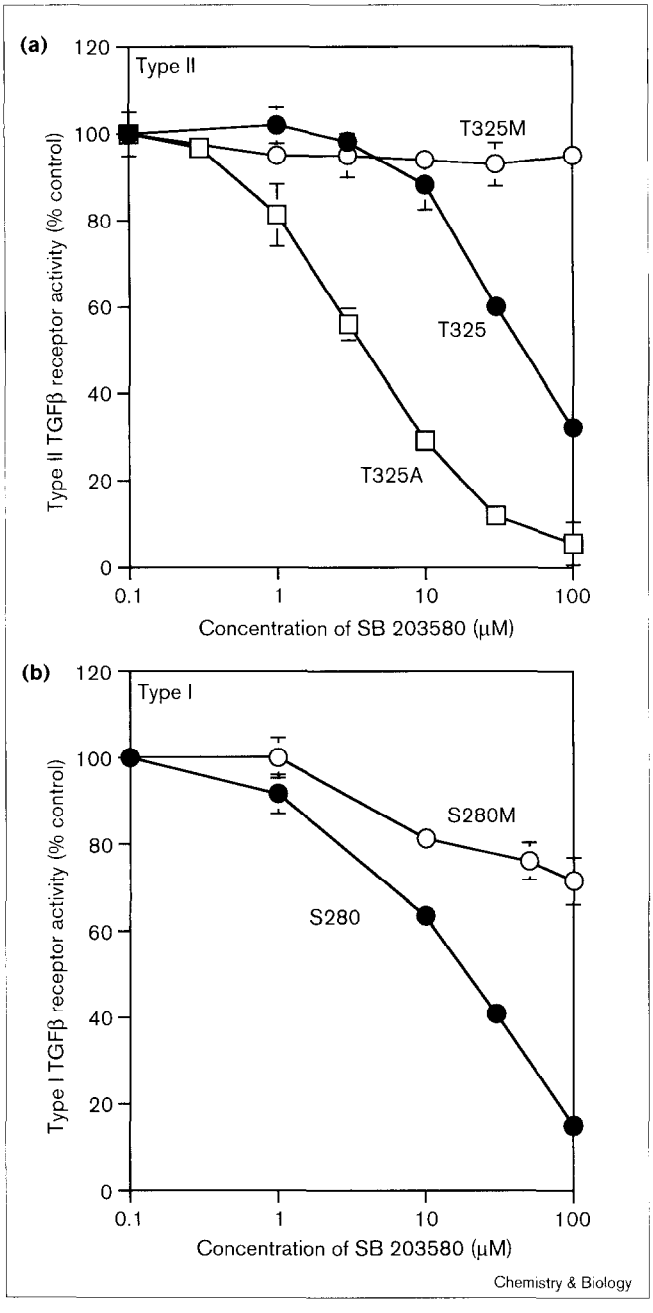
Thr106 of SAPK2a/p38 is located in subdomain IV of the kinase catalytic domain (Figure 1). Examination of the sequences of other protein kinases revealed that a bulky hydrophobic residue is almost always found at this position. Nevertheless, a small number of protein kinases do have threonine at this position, such as the type II transforming growth factor β (TGFβ) receptor (a serine/threonine protein kinase), members of the Src family of protein tyrosine kinases and some receptor protein tyrosine kinases, such as the epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptors (Figure 1). In order to determine if the presence of threonine at this position is diagnostic of sensitivity to SB 203580, we examined whether the type II TGFβ receptor [23] and Lck (a Src family member) [24] were inhibited by this drug. These experiments revealed that both kinases were inhibited by SB 203580, with IC_{50} values of 20 μM (Lck) and 40 μM (type II TGFβ receptor) (Figure 4a). When Thr325 in the type II TGFβ receptor was changed to methionine, the enzyme became insensitive to the drug (Figure 4a). By contrast, when Thr325 was changed to alanine, the type II TGFβ receptor was inhibited by SB 203580 with an IC_{50} of 4 μM (Figure 4a). In addition, SK&F 105809, a closely related pyridinyl imidazole that does not inhibit SAPK2a/p38 [5], did not inhibit any of the type II TGFβ receptor constructs (data not shown). The type II TGFβ receptor was inhibited by SB 203580 with the same IC_{50} value whether it was assayed using autophosphorylation (Figure 4a) or using histone H2B as a substrate (data not shown). These results establish that the sensitivity of the type II TGFβ receptor to SB 203580 is conferred by Thr325 in subdomain IV.

Figure 3



Inhibition of wild-type and mutant SAPK1γ/JNK1 by SB 203580. The effect of the drug on the wild-type enzyme is shown by the closed circles and its effect on the mutant enzymes by open symbols. Mutations are designated using single-letter amino-acid code. Wild-type SAPK1γ/JNK1 is resistant to SB 203580, because it has a methionine residue at position 108 and an isoleucine residue at position 106. The kinase becomes sensitive to SB 203580 when Met108 is mutated to threonine, and even more sensitive when Met108 is mutated to alanine.

Figure 4



Inhibition of the type I TGFβ receptor and the type II TGFβ receptor by SB 203580. **(a)** The effect of SB 203580 on the native type II TGFβ receptor is shown by closed circles and its effect on the mutant enzymes by open symbols. The type II TGFβ receptor has a threonine residue at position 325 and is inhibited by SB 203580 with an IC_{50} value of 40 μM. The Thr325→Ala mutant is inhibited with an IC_{50} value of 4 μM, whereas the Thr325→Met mutant is resistant to SB 203580. **(b)** The effect of SB 203580 on the native type I TGFβ receptor is shown by closed circles and its effect on the Ser280→Met mutant by open circles. The type I TGFβ receptor has a serine residue at position 280 and is inhibited by SB 203580 with an IC_{50} value of 20 μM. Replacement of this serine residue with methionine increases the IC_{50} value to > 100 μM. Mutations are designated using single-letter amino-acid code.

Inhibition of the type I TGFβ receptor by SB 203580

Very few protein kinases have serine at the equivalent position of Thr106 of SAPK2a/p38 (for instance a mammalian type I TGFβ receptor [25], SAPK2a/p38 and SAPK2b/p38β2 from *Caenorhabditis elegans* [Genbank accession numbers: 1326389 and 2702444] and MAP kinase from *Dictyostelium discoideum* [26]; Figure 1). In order to determine if the presence of serine at this position confers sensitivity to SB 203580, we examined whether the type I TGFβ receptor was inhibited by this compound. As shown in Figure 4b, the type I TGFβ receptor was sensitive to inhibition by SB 203580, with an IC_{50} value of 20 μM. By contrast, when Ser280 in the type I TGFβ receptor was changed to methionine, the enzyme became insensitive to the drug. These findings are consistent with the mutagenesis study on SAPK3 which showed that the Met109→Ser mutant was more sensitive to inhibition by SB 203580 than the Met109→Thr mutant (Table 1).

Discussion

The present findings demonstrate that inhibition of SAPK2a/p38 and SAPK2b/p38β2 by the pyridinyl imidazole SB 203580 depends on the size of the amino-acid residue at position 106 of the ATP-binding region. Mutation of residue 106 from threonine to methionine made SAPK2b/p38β2 insensitive to SB 203580. It also rendered SAPK2a/p38 almost completely resistant to SB 203580, in agreement with a previous study [22]. Conversely, mutation of the equivalent residue in SAPK3 and SAPK4 from methionine to threonine rendered these enzymes sensitive to inhibition by SB 203580. Further mutagenesis revealed that SAPK3/p38γ became more sensitive to the drug when methionine was replaced by even smaller residues, such as serine, alanine or glycine. SAPK2a/p38, SAPK2b/p38β2 and SAPK4/p38δ also became most sensitive to SB 203580 when glycine or alanine was present at this position. These findings rule out the possibility that the inhibition of SAPK2a/p38 and SAPK2b/p38β2 results from the formation of a hydrogen bond between the hydroxyl group of Thr106 and the 4-fluorophenyl moiety of SB 203580. Although SAPK1/JNK, another MAP kinase family member, is only 40% identical to SAPK2a/p38 and SAPK2b/p38β2 [13] it could also be converted to a SB 203580-sensitive form by mutation of Met108 to a small amino acid. In order to optimise sensitivity to the drug, however, it was also necessary to change Ile106 to the leucine residue present at the equivalent position in other MAP kinase family members. The latter result could explain the reported inhibition of JNK2β1 and JNK2β2 by high concentrations of a pyridinyl imidazole compound [27], as these isoforms of SAPK1/JNK have leucine at position 106 and methionine at position 108 [13].

Most known mammalian protein kinases have a large, hydrophobic residue at the position equivalent to Thr106 of SAPK2a/p38. Few protein kinases have threonine at

this position, and only two (the type I TGFβ receptor and a type I activin receptor) have serine at this position. Two protein kinases that have threonine at the position equivalent to the SAPK2a/p38 Thr106 (the type II TGFβ receptor and the tyrosine protein kinase Lck) were found to be sensitive to inhibition by SB 203580, although the IC_{50} values were 400–800 times higher than the IC_{50} value for SAPK2a/p38, indicating that residues other than the one equivalent to Thr106 of SAPK2a/p38 are involved in sensitivity determination. Nevertheless, sensitivity of the type II TGFβ receptor to SB 203580 was enhanced by mutagenesis of Thr325 to alanine, whereas it was abolished when Thr325 was changed to methionine. The type I TGFβ receptor was inhibited more potently by SB 203580 than the type II TGFβ receptor, consistent with the presence of the smaller serine residue at the position equivalent to Thr106 of SAPK2a/p38.

When studying phenotypic effects of SB 203580 in cells, we have previously emphasized the importance of establishing that these effects occur at a concentration similar to that which prevents the activation of SAPK2a/p38 in the same cell type [7]. The realisation that other protein kinases that contain serine or threonine at position 106 equivalent are inhibited by SB 203580 at concentrations above 10 μM makes this control mandatory. It will also be important to determine if all the reported effects of SB 203580 in mammalian cells are abolished by transfection with a drug-insensitive form of SAPK2a/p38 or by the use of transgenic mice expressing an SB 203580-insensitive form of the SAPK2/p38 protein kinase.

No known protein kinase has glycine or alanine at the position equivalent to Thr106 of SAPK2a/p38. If such enzymes exist, they can be expected to be potently inhibited by SB 203580. Recent work on non-receptor tyrosine kinases of the Src family has shown that the size of the residue equivalent to Thr106 in SAPK2a/p38 is also primarily responsible for determining the ability of protein kinases to accept N6-substituted ATP analogues [28], further emphasized the crucial role that this residue plays in determining the size of the ATP-binding pocket of protein kinases.

Nearly all eukaryotic kinases belong to the same protein superfamily and their ATP-binding sites are similar. It therefore seemed unlikely that specific inhibitors of particular protein kinases could be developed by targeting the ATP-binding region of these enzymes. Recent work describing a new class of receptor protein tyrosine kinase inhibitors suggests that this fear may be unfounded [29]. In addition, the present work indicates that remarkable specificity can be achieved if drugs that interact with the ATP-binding pocket carry an additional moiety that makes contact with residues outside this region. Our finding that sensitivity to SB 203580 can be gained by single amino-acid substitutions means that it should be possible to use the same drug to identify the physiological roles of all MAP kinase family members and perhaps other protein kinases. Replacement of wild-type SAPK1/JNK isoforms, SAPK3 or SAPK4 by drug-sensitive forms of these enzymes in transgenic mice expressing a drug-resistant form of SAPK2a/p38 could also be useful in addressing the physiological roles of these MAP kinase family members.

Finally, our results show that it is possible to predict whether or not a protein kinase will be sensitive to inhibition by this class of pyridinyl imidazoles simply by inspection of the amino acid at the position equivalent to Thr106 of SAPK2a/p38 in subdomain IV of the kinase catalytic domain. It might, therefore, be possible to identify lead inhibitors for some protein kinases without any high-throughput screening.

Significance

Protein kinases have important roles in many physiological processes, and they have become attractive targets for drug therapy because abnormal protein phosphorylation is an essential feature of many disease states. It is widely believed that specific protein-kinase inhibitors will constitute an important class of future drugs, permitting improved treatment of a large number of serious diseases, including cancer, diabetes and hypertension.

It is, therefore, critical to understand the mechanisms of action of the few existing protein kinase inhibitors that have a high degree of specificity. The pyridinyl imidazole SB 203580 is one such inhibitor. It inhibits stress-activated protein (SAP) kinase-2a (SAPK2a, also called p38) and SAP kinase-2b (SAPK2b, also called p38β2), but not other members of the mitogen-activated protein (MAP) kinase family. SB 203580 has been used to identify some of the physiological substrates and cellular functions of SAPK2a/p38 and SAPK2b/p38β2. Here we show that for a protein kinase to be sensitive to SB 203580, the amino acid at the position equivalent to residue 106 of SAPK2a/p38 must be no larger than threonine, the sensitivity being greatly enhanced when this residue is even smaller, such as serine, alanine or glycine. The high degree of specificity of SB 203580 results from the presence of a residue larger than threonine at this position in nearly all known protein kinases. These findings explain how drugs that target the ATP-binding site can inhibit protein kinases specifically and show that it is possible to predict whether or not a protein kinase will be sensitive to inhibition by this class of pyridinyl imidazoles simply by inspection of the amino acid at the position equivalent to Thr106 of SAPK2a/p38 in subdomain IV of the kinase catalytic domain. Our data should help to facilitate the rational design of specific protein-kinase inhibitors.

Materials and methods

Site-directed mutagenesis and expression of protein kinases

The open reading frame of human SAPK2a/p38 (CSBP2 isoform) [4] was amplified by PCR from brain cDNA, verified by DNA sequencing and subcloned into M13. Full-length cDNA clones encoding human SAPK1γ/JNK1, human SAPK2b/p38β2, rat SAPK3 and human SAPK4 were also subcloned into M13. Site-directed mutagenesis was used to change Ile106 in SAPK1γ/JNK1 to leucine and/or Met108 to alanine or threonine; Thr106 in SAPK2a/p38 was changed to alanine, glycine or methionine; Thr106 in SAPK2b/p38β2 was changed to alanine, glycine or methionine; Met109 in SAPK3 was changed to alanine, glutamine, glutamate, glycine, leucine, lysine, phenylalanine, serine or threonine; Met107 in SAPK4 was changed to alanine, glycine or threonine. All mutations were verified by DNA sequencing. Following primer extension, the mutated cDNAs were subcloned into bacterial expression vectors pRSETB (Invitrogen) or pGEX4T-1 (Pharmacia) and expressed as His-tagged proteins (SAPK1γ/JNK1) or as GST-fusion proteins (SAPK2a/p38, SAPK2b/p38β2, SAPK3 and SAPK4) in *Escherichia coli* BL21 (DE3) cells. Human Lck [24] was expressed in Sf9 cells from a recombinant baculoviral vector. Site-directed mutagenesis was used to change Thr325 in the type II TGFβ receptor [23] to methionine or alanine. Mutagenesis was carried out as described previously [30]. The carboxy-terminal 375 residues of the wild-type TGFβ type II receptor and the corresponding mutants were expressed in *E. coli* as GST-fusion proteins and purified on glutathione-Sepharose [31]. Site-directed mutagenesis was used to change Ser280 in the type-I TGFβ receptor [25] to methionine. The carboxy-terminal 356 residues of the wild-type and mutant type I TGFβ receptors were expressed in *E. coli* as GST-fusion proteins and purified on glutathione–sepharose.

Protein kinase assays

Wild-type SAPK1γ/JNK1 and its mutants were activated using bacterially expressed SAPK kinase-1 (SKK1, also called MKK4 or SEK1), which had been activated by MEK kinase-1 and assayed at 30°C in the presence and absence of the indicated concentrations of SB 203580 using GST–ATF2(19–96) as substrate. Wild-type SAPK2a/p38, SAPK2b/p38β2, SAPK3 and SAPK4 and their respective mutants were purified on glutathione–sepharose and activated by a partially active mutant of human SAPK kinase-3 (SKK3, also called MKK6), in which Ser274 and Thr278 had been mutated to aspartate. This constitutively active SKK3/MKK6 mutant was expressed as a maltose-binding protein fusion protein in *E. coli* and purified on an amylose resin. Each SAP kinase was assayed at 30°C in the presence and absence of the indicated concentrations of SB 203580 using myelin basic protein as substrate, as described previously for MAPK2/ERK2 kinase [32]. Mutants of SAPK1γ/JNK1, SAPK2a/p38, SAPK2b/p38β, SAPK3 and SAPK4 were activated at the same rates and to the same specific activities as the wild-type enzymes. Human Lck was assayed using the peptide KVEKIGEGTYGVVYK, in the presence and absence of the indicated concentrations of SB 203580. The type II TGFβ receptor and its mutants were assayed at 30°C in the presence and absence of SB 203580 or SK&F 105809 by the rate of autophosphorylation or using histone H2B (0.1 mg/ml) as substrate. The mutants of the type II TGFβ receptor had the same specific activity as the wild-type enzyme. The type I TGFβ receptor was assayed by the rate of autophosphorylation at 30°C in the presence or absence of the indicated concentrations of SB 203580. The concentration of ATP in all assays was 0.1 mM. SB 203580 was purchased from Calbiochem.

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