The signal-inducible phosphorylation of serines 32 and 36 of IκBα is critical in regulating the subsequent ubiquitination and proteolysis of IκBα, which then releases NF-κB to promote gene transcription. The multisubunit IκB kinase responsible for this phosphorylation contains two catalytic subunits, termed IκB kinase (IKK)-1 and IKK-2. BMS-345541 (4(2’-aminoethyl)amino-1,8-dimethylimidazol(1,2-a)quinoxaline) was identified as a selective inhibitor of the catalytic subunits of IKK (IKK-2 IC_{50} = 0.3 \mu M, IKK-1 IC_{50} = 4 \mu M). The compound failed to inhibit a panel of 15 other kinases and selectively inhibited the stimulated phosphorylation of IκBα in cells (IC_{50} = 4 \mu M) while failing to affect c-Jun and STAT3 phosphorylation, as well as mitogen-activated protein kinase-activated protein kinase 2 activation in cells. Consistent with the role of IKK/NF-κB in the regulation of cytokine transcription, BMS-345541 inhibited lipopolysaccharide-stimulated tumor necrosis factor α, interleukin-1β, interleukin-8, and interleukin-6 in THP-1 cells with IC_{50} values in the 1- to 5-μM range. Although a Dixon plot of the inhibition of IKK-2 by BMS-345541 showed a non-linear relationship indicating non-Michaelis-Menten kinetic binding, the use of multiple inhibition analyses indicated that BMS-345541 binds in a mutually exclusive manner with respect to a peptide inhibitor corresponding to amino acids 26–42 of IκBα with Ser-32 and Ser-36 changed to aspartates and in a non-mutually exclusive manner with respect to ADP. The opposite results were obtained when studying the binding to IKK-1. A binding model is proposed in which BMS-345541 binds to similar allosteric sites on IKK-1 and IKK-2, which then affects the active sites of the subunits differently. BMS-345541 was also shown to have excellent pharmacokinetics in mice, and peroral administration showed the compound to dose-dependently inhibit the production of serum tumor necrosis factor α following intraperitoneal challenge with lipopolysaccharide. Thus, the compound is effective against NF-κB activation in mice and represents an important tool for investigating the role of IKK in disease models.

The expression of many pro-inflammatory genes is regulated by the transcriptional activator NF-κB. Genes dependent on activation of NF-κB include the cytokines TNFα, IL-6, IL-8, and IL-1β; the adhesion molecules E-selectin, ICAM-1, and VCAM-1; and the enzymes nitric-oxide synthase and COX-2 (for reviews see Refs. 1 and 2). NF-κB normally resides in the cytoplasm of unstimulated cells as an inactive complex with a member of the IκB inhibitory protein family. This class of protein includes IκBα, IκBβ, and IκBε, which all contain ankyrin repeats necessary to form a complex with NF-κB (for a review see Ref. 3). In the case of IκBα, the most carefully studied member of this class, stimulation of cells with agents that activate NF-κB-dependent gene transcription results in the phosphorylation of IκBα at Ser-32 and Ser-36 (4). This is critical for subsequent ubiquitination and proteolysis of IκBα, which then leaves NF-κB free to translocate to the nucleus and promote gene transcription (5–7). Indeed, a mutant in which both Ser-32 and Ser-36 have been changed to alanine prevents signal-induced activation of NF-κB and results in an IκBα that is not phosphorylated, ubiquitinated, or digested proteolytically (7). Analogous serines have been identified in both IκBβ and IκBε, and phosphorylation at these residues appears to regulate the proteolytic degradation of these proteins by a mechanism similar to that of IκBα (8, 9).

A high molecular mass (500–900 kDa) multisubunit IκB kinase (termed IKK) that phosphorylates at Ser-32 and Ser-36 of IκBα has been isolated from HeLa cells (10–12). Two catalytic subunits (termed IKK-1 and IKK-2) of IKK have recently been identified, cloned, and shown to be widely expressed in human tissues (12–17). The use of gene-targeting experiments have clearly shown that all known proinflammatory stimuli, including cytokines, viruses, and lipopolysaccharide (LPS) require the IKK-2 subunit for NF-κB activation (for a review see Ref. 18). Although the role of IKK-1 in NF-κB activation is still unclear, recent evidence suggests that IKK-1 may only play a role in response to certain stimuli (e.g. RANK-ligand and Blys/BAFF) and in select cells such as mammary epithelial cells and B lymphocytes (19).

Given the importance of NF-κB in regulating inflammatory processes, the identification of selective IKK inhibitors has
Inhibition of IKK by BMS-345541

EXPERIMENTAL PROCEDURES

Materials—GST-IkB-a was purchased from Santa Cruz Biotechnol-
ology, and [32P]ATP (1000 Ci/mmol) was purchased from Amesher Biosciences, and ADP, EGF, and staurosporine were obtained from Sigma-Aldrich. The amount of HPLC grade solvent dichloromethane (DCM) was 26–42 of I kB-a (LDDDDGGLDSMKDEY, N-terminal acetylated and C-terminal amidated), along with a peptide inhibitor that corresponds to the same amino acids except with Ser-32 and Ser-36 changed to Gln-32 and Gln-36 corresponding to amino acids 26–42 of I kB-a as described previously (21, 28). In this assay, either IKB-1 or IKB-2 was added at 30 °C to solutions containing peptide and [32P]ATP (1000 Ci/mmol) in 50 mM Tris-HCl, 5 mM MgCl2 at pH 8. After 60 min, the kinase reactions were quenched by addition of EDTA to a concentra-
tion of 10 mM. HPLC analysis was performed as described previously (21) for stepwise dephosphorylation to determine the extent of dephosphorylation. The extent of peptide was quantitated by liquid scintillation counting. Under these conditions, the degree of phosphorylation of peptide substrate was linear with time and concentration of enzyme.

Because the above-described SDS-PAGE assay is not sufficiently precise for kinetic analyses of the IKK enzymes, assays for enzyme kinetic studies instead measured the enzyme-catalyzed phosphoryla-
tion of 4(2'-aminoethylamino)-1,8-dimethylimi-
dazo(1,2-a)quinoxaline as a highly selective inhibitor of IKK that inhibits NF-kB-dependent transcription of pro-inflammatory cytokines both in vitro and in vivo. The compound appears to bind to an unidentified allosteric binding site of the IKK catalytic subunits.

Received considerable interest. We report here the identifica-
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Inhibition of IKK by BMS-345541

RESULTS

Using an assay measuring the IKK-2-catalyzed phosphorylation of GST-IxBα, BMS-345541 was identified as an inhibitor of the enzyme. As shown in Fig. 1, BMS-345541 dose-dependently inhibited IKK-2 with an IC_{50} value of ~0.3 μM. The compound was considerably less potent against IKK-1 with an IC_{50} value of 4 μM and did not inhibit the related kinase IKK-ε. When tested against a panel of both serine/threonine and tyrosine kinases such as human recombinant protein kinase Ca, protein kinase Cθ, protein kinase Cδ, protein kinase Cε, protein kinase A, Her1, Her2, p38α, MAPKAP K2, JAK3, EMT kinase, lck, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase, extracellular signal-regulated kinase 1/2, and insulin-like growth factor-1R, BMS-345541 (Structure I) failed to inhibit any kinase at concentrations as high as 100 μM (results not shown).

When assayed in THP-1 monocytic cells, the compound dose-dependently inhibited the TNFα-stimulated phosphorylation of IxBα with an IC_{50} value of ~4 μM (Fig. 2A). The compound was not cytotoxic in this concentration range as determined by trypan blue exclusion (results not shown). Consistent with the compound being a highly selective inhibitor of IKK, BMS-345541 at concentrations as high as 100 μM failed to block both the anisomycin-stimulated phosphorylation of c-Jun and LPS-stimulated activation of MAPKAP K2 in THP-1 cells, as well as the EGF-stimulated phosphorylation of STAT3 in H292 cells. As shown in Fig. 2B, the compound was also effective in THP-1 cells at inhibiting the stimulated production of a number of cytokines from THP-1 cells including TNFα, IL-1β, IL-8,
Inhibition of IKK by BMS-345541

**Inhibitor of IKK-2.** The IKK-2-catalyzed phosphorylation of the peptide corresponding to amino acids 26–42 of IκBα was measured. See “Experimental Procedures” for details.

and IL-6. The IC_{50} against cytokine production in these cells fell in the range of 1–5 μM, which matched the inhibition observed against IκBα phosphorylation. This observation is consistent with both the essential requirement of NF-κB in promoting the transcription of these cytokines and the important role of IKK in regulating NF-κB transcription.

**Enzyme Kinetic Analysis of Inhibition**—To investigate the binding mechanism of BMS-345541 to IKK-2, a simple analysis of whether the inhibitor was competitive with respect to ATP or peptide substrate was undertaken. However, non-linear fits of the data to both competitive and mixed non-competitive inhibition were very poor, and a definitive answer could not be obtained (results not shown). The reason for these poor fits is that the inhibitor shows non-linear binding kinetics. Indeed, Fig. 3 shows that BMS-345541 does not give a linear Dixon plot of [Inhibitor] versus rate \(^{-1}\). For an inhibitor that follows simple Michaelis-Menten kinetics, a linear correlation would be expected for competitive, non-competitive, mixed, and uncompetitive inhibitors. This non-linearity does not appear to result from solubility problems, because the compound is very water-soluble. Moreover, the Dixon plot of the inhibition of IKK-1 by BMS-345541 is more linear even at concentrations nearly 100-fold higher (results not shown), which indicates the effect is dependent on IKK-2.

As an alternative way to investigate the binding mechanism, the effect of BMS-345541 on the inhibition kinetics of ADP and a peptide inhibitor corresponding to amino acids 26–42 of IκBα with Ser-32 and Ser-36 changed to aspartates was determined. Not surprisingly, ADP has been shown to be a competitive inhibitor with respect to the ATP substrate (24), and the peptide inhibitor is a competitive inhibitor with respect to peptide substrate (20). As shown in Fig. 4A, a Dixon plot of the inhibition of IKK-2 by ADP at different fixed concentrations of peptide inhibitor gave non-parallel relationships. At each fixed concentration of inhibitor, the value of kinetic constant \(K_s\), which is directly proportional to the slope of the Dixon plots, were calculated by a non-linear fit of the data to be 775 ± 75, 1952 ± 229, 3286 ± 212, and 4564 ± 347 s\(^{-1}\)M\(^{-1}\) at 0, 157, 314, and 471 μM peptide inhibitor, respectively. Non-parallel lines (i.e., increasing values of \(A\)) in this type of multiple inhibition analysis is the hallmark of non-mutually exclusive inhibitors and means that both inhibitors can bind to the enzyme simultaneously (25). This conclusion is expected, because the ATP and peptide binding sites of kinases are distinct regions of the active site, which allows both substrates (or inhibitors in the case of ADP and peptide inhibitor) to be bound simultaneously. Indeed, both ATP and peptide substrates are required to bind simultaneously for kinases to function as catalysts.

A similar relationship exists between BMS-345541 and ADP as shown in Fig. 4B. Indeed, the values for kinetic constant \(A\) at different fixed concentrations of BMS-345541 were found to be 853 ± 88, 1180 ± 203, 1287 ± 144, 1617 ± 104, and 2021 ± 207 s\(^{-1}\)M\(^{-1}\) at 0, 0.5, 1, 1.5, and 2 μM BMS-345541, respectively. The resulting non-parallel lines indicate that BMS-345541 and ADP bind to different sites on IKK-2. Therefore, BMS-345541 does not bind to the ATP binding site.

Confirmatory evidence in support of this conclusion comes from the third permutation of this multiple inhibition analysis: the effect of BMS-345541 on the inhibition of peptide substrate. As shown in Fig. 4C, the Dixon plots gave essentially parallel relationships, which reflects the relatively unchanging values of kinetic constant \(A\) at different fixed concentrations of BMS-345541: 103 ± 7, 101 ± 2, 99 ± 4, 109 ± 2, and 119 ± 6 s\(^{-1}\)M\(^{-1}\) at 0, 0.3, 0.7, 1.3, and 2 μM BMS-345541, respectively. Parallel relationships in this type of Dixon plot are indicative of mutually exclusive inhibitors in which both BMS-345541 and peptide inhibitor cannot bind to IKK-2 simultaneously (26). Although this may suggest that BMS-345541 and peptide inhibitor substrate bind at the same site, mutually exclusive inhibition also can result from inhibitors that bind at different sites but in a mutually exclusive manner (e.g., occupation at one site induces a protein conformational change that affects and prevents binding at the other site).

With IKK-1, ADP and peptide inhibitor showed the same relationship as seen with IKK-2. That is, non-parallel Dixon plots of the inhibition of ADP at different fixed concentrations of peptide inhibitor (see Fig. 5A) with the values for kinetic constant \(A\) determined to be 345 ± 13, 1300 ± 74, 2200 ± 81, and 2771 ± 114 s\(^{-1}\)M\(^{-1}\) at 0, 423, 471, and 518 μM peptide inhibitor, respectively. As with IKK-2, non-parallel relationships indicates that ADP and peptide inhibitor bind at different sites to IKK-1 and can bind simultaneously.

Unlike the result with IKK-2, however, Fig. 5B indicates that BMS-345541 and peptide inhibitor bind to IKK-1 in a non-mutually exclusive manner (i.e. can bind to the enzyme at the same time). Indeed, the Dixon plot showed non-parallel relationships with values for kinetic constant \(A\) determined to be 112 ± 5, 233 ± 37, 284 ± 24, and 446 ± 57 at s\(^{-1}\)M\(^{-1}\).
Characterization of BMS-345541 in Vivo—Because BMS-345541 is both highly selective for IKK and potent against stimulated activation of NF-κB-dependent gene transcription in cells, the biological activity in vivo was investigated. As shown in Fig. 6, peroral administration of BMS-345541 at a concentration of 10 mg/kg to mice resulted in prolonged serum drug levels, with concentrations sustained at 29, 477 ± 42, 487 ± 57, and 486 ± 78 s counts⁻¹·M⁻¹ at 0, 35, 70, 105, and 140 μM BMS-345541, respectively. This indicates that BMS-345541 and ADP are mutually exclusive inhibitors in which both cannot bind to IKK-1 simultaneously. This is in contrast to IKK-2 where ADP and BMS-345541 were shown to be non-mutually exclusive inhibitors.

Inhibition of IKK by BMS-345541

at 0, 471, 518, and 565 μM peptide inhibitor, respectively. A similar dichotomy between IKK-2 and IKK-1 was also observed using BMS-345541 and ADP in a multiple inhibition analysis as shown in Fig. 5C. In this case, the Dixon plots gave essentially parallel relationships that reflect the relatively unchanged values of kinetic constant A for the inhibition by ADP at different fixed concentrations of BMS-345541: 439 ± 4, 439 ± 2, 439 ± 3, 439 ± 4, and 439 ± 5 s counts⁻¹·M⁻¹ at 0, 35, 70, 105, and 140 μM BMS-345541, respectively. This indicates that BMS-345541 and ADP are mutually exclusive inhibitors in which both cannot bind to IKK-1 simultaneously. This is in contrast to IKK-2 where ADP and BMS-345541 were shown to be non-mutually exclusive inhibitors.

Characterization of BMS-345541 in Vivo—Because BMS-345541 is both highly selective for IKK and potent against stimulated activation of NF-κB-dependent gene transcription in cells, the biological activity in vivo was investigated. As shown in Fig. 6, peroral administration of BMS-345541 at a concentration of 10 mg/kg to mice resulted in prolonged serum drug levels, with concentrations sustained at...
or above 1 μM for many hours. By comparing the area under the curves for the peroral administration and intravenous legs, an oral bioavailability of ~100% was determined. The results from this study were used to obtain the pharmacokinetic parameters shown in Table I. Additional studies showed that the drug levels and total exposure after peroral administration of BMS-345541 were proportional at doses up to 100 mg/kg (results not shown).

Because of the excellent pharmacokinetics displayed by BMS-345541, its biological activity in mice was investigated. As shown in Fig. 7, BMS-345541 dose-dependently inhibited the production of TNFα measured in the serum of animals challenged with an intraperitoneal administration of LPS. Approximately 50% inhibition was observed at 10 mg/kg, consistent with the micromolar exposure shown in Fig. 6 and the cell potency shown in Fig. 2. Near complete inhibition of serum TNFα was observed at a dose of 100 mg/kg BMS-345541, with six of eight animals in the group showing undetectable serum TNFα levels.

**DISCUSSION**

We have shown that BMS-345541 is highly selective for the IKK catalytic subunits versus numerous other kinases. This selectivity is also evident in cells; only the stimulus-induced phosphorylation of IκBα was inhibited by BMS-345541 whereas other signal transduction cascades were unaffected. This is especially important, because there is a cascade of kinases working sequentially in many of these signaling pathways such that blocking any of the upstream kinases would have resulted in inhibition of the measured end point (e.g., inhibition of c-Jun NH₂-terminal kinase, SEK, or mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase, etc. would have inhibited c-Jun phosphorylation). Lack of effects on these signal transduction pathways in cells, therefore, provides additional evidence of the selectivity of BMS-345541 for the catalytic subunits of IKK.

Between IKK-1 and IKK-2, BMS-345541 showed approximately an order-of-magnitude selectivity for the latter. Having selectivity for IKK-2 over IKK-1 may be advantageous from the standpoint of the toxicity profile of a therapeutic agent, because IKK-2 has been shown to play the critical role in IκB activation and NF-κB activation in response to pro-inflammatory stimuli whereas IKK-1 may play only a minor role (for a review, see Ref. 18). Indeed, IKK-1 is involved in keratinocyte differentiation, although this appears not to be dependent on its kinase activity or role in NF-κB activation (27).

The use of multiple inhibition analyses with IKK-2 indicated that BMS-345541 bound in a non-mutually exclusive manner with respect to ADP and a mutually exclusive manner with respect to peptide inhibitor. This suggests that BMS-345541 binds not to the ATP binding site but to either the peptide binding site or an allosteric site, which, when occupied, alters the peptide binding site and prevents peptide binding. Curiously, the exact opposite relationship was observed with multiple inhibition analyses using IKK-1. The results are consistent with one of the following two possible binding mechanisms. 1) BMS-345541 binds to the ATP binding site of IKK-1 but not of IKK-2, or 2) BMS-345541 binds to similar allosteric sites on IKK-1 and IKK-2, but binding to the allosteric site on IKK-2 leads to a conformational change in the enzyme that alters the peptide binding site whereas the conformational change that occurs upon binding of BMS-345541 to the corresponding site on IKK-1 causes a perturbation of the ATP binding site. It is difficult to differentiate between these two possibilities without crystallographic evidence. However, structure-activity relationships with numerous other inhibitors synthesized in this chemical class show the same -fold selectivity for IKK-2 for IKK-1 across a wide range of potencies (results not shown). If BMS-345541 bound to a different site on IKK-2 than on IKK-1, the structure-activity relationships would not be expected to track between IKK-1 and IKK-2. Therefore, it is likely that BMS-345541 binds to similar allosteric sites on IKK-1 and IKK-2 but that the conformational change that then affects the active site is somewhat different (i.e., mechanism 2 above).

**FIG. 6. Pharmacokinetics of BMS-345541 in the mouse.** Concentration [μM] of BMS-345541 in serum after a 2 mg/kg intravenous dose (closed circles) and a 10 mg/kg peroral dose (open triangles) in mice. n = 3 animals per dosing route with the error bars representing standard deviations.

**FIG. 7. The effect of BMS-345541 on serum TNFα concentrations induced by intraperitoneal injection of LPS.** BMS-345541 was administered perorally 60 min prior to LPS challenge, and blood was drawn 90 min subsequent to challenge. n = 8 animals per group with the error bars representing standard deviations. Note: one animal in the 30 mg/kg dose group and six animals in the 100 mg/kg dose group had serum TNFα levels less than the level of detection in the assay (i.e., <50 pg/ml). For the purposes of plotting, these animals were assigned a value of 50 pg/ml.

**TABLE I**

| Summary of mean pharmacokinetic parameters for BMS-345541 |
|----------------------------------------------------------|
| An aqueous solution of BMS-345541 was administered by oral (10 mg/kg) or intravenous (2 mg/kg) routes to the mouse. Cₘₐₓ (p.o.) is defined as the maximum concentration achieved after peroral administration; Tₘₐₓ (p.o.) is the time after administration that the Cₘₐₓ is obtained, and Vₘₐₓ is the apparent steady-state volume of distribution. |
| parameter          | value             |
|---------------------|-------------------|
| Cₘₐₓ (p.o.)         | 1.6 ± 0.44 μM     |
| Tₘₐₓ (p.o.)         | 0.52 h            |
| Intravenous half-life | 2.2 ± 0.52 h     |
| Vₘₐₓ                | 18.5 ± 1.5 liter/kg |
| Total clearance     | 113.5 ± 17.6 ml · min⁻¹ · kg⁻¹ |
That BMS-345541 binds to an allosteric site on IKK-2 and IKK-1 is also consistent with the high degree of selectivity for the IKK catalytic subunits versus other kinases. Indeed, most kinase inhibitors bind to the ATP binding site, which, because the site is highly conserved among kinases, makes it difficult to obtain selective inhibitors. The high selectivity of BMS-345541 for IKK-2 and IKK-1 suggests that the putative allosteric site is unique to the IKKs, although the site may be present within other kinases not tested for selectivity.

It has been reported recently (27) that the catalytic subunits IKK-2 and IKK-1, which are expressed as dimers, contain non-equivalent binding sites acting in a cooperative fashion such that binding of substrate or inhibitor at one active site affects the conformation at the other active site. The non-linear kinetics observed with BMS-345541 binding to IKK-2 are also consistent with this model. From the relationship shown in Fig. 3, it would appear that either the two active sites within the IKK-2 homodimer are non-equivalent and bind BMS-345541 with different affinities, or the two active sites are equivalent when unoccupied but that binding of BMS-345541 to one active site within the dimer adversely affects binding of BMS-345541 to the second site. Although this certainly adds a layer of complexity to the analysis of inhibitor binding studies, the multiple inhibition analyses detailed in the present work demonstrate that questions about binding sites/mechanisms of the IKK catalytic subunits can be answered.

An especially important characteristic of BMS-345541 is the excellent pharmacokinetic profile in mice. Complete absorption after peroral administration, coupled with a very long intravenous half-life, results in blood levels that should be sufficient to inhibit IKK \textit{in vivo} (e.g. BMS-345541 inhibits IKK in cells in the micromolar range, and a 10 mg/kg peroral administration dose gives micromolar levels of drug for many hours). Indeed, BMS-345541 dose-dependently inhibited LPS (intraperitoneal)-induced serum TNF\textalpha production in mice at doses in the 3 to 100 mg/kg range. To our knowledge, BMS-345541 represents the first selective IKK inhibitor reported with \textit{in vivo} activity. The potency and selectivity of BMS-345541 described herein, allied with an excellent pharmokinetics profile, makes this compound ideally suited to probe the role of IKK \textit{in vivo}. Work is ongoing in our laboratory to evaluate BMS-345541 in multiple murine models of human disease.

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