Monocarbonyl Curcumin Analogues: Heterocyclic Pleiotropic Kinase Inhibitors That Mediate Anticancer Properties

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ABSTRACT: Curcumin is a biologically active component of curry powder. A structurally related class of mimetics possesses similar anti-inflammatory and anticancer properties. Mechanism has been examined by exploring kinase inhibition trends. In a screen of 50 kinases relevant to many forms of cancer, one member of the series (4, EF31) showed ≥85% inhibition for 10 of the enzymes at 5 μM, while 22 of the proteins were blocked at ≥40%. IC₅₀ values for an expanded set of curcumin analogues established a rank order of potencies, and analyses of IKKβ and AKT2 enzyme kinetics for 4 revealed a mixed inhibition model, ATP competition dominating. Our curcumin mimetics are generally selective for Ser/Thr kinases. Both selectivity and potency trends are compatible with protein sequence comparisons, while modeled kinase binding site geometries deliver a reasonable correlation with mixed inhibition. Overall, these analogues are shown to be pleiotropic inhibitors that operate at multiple points along cell signaling pathways.

INTRODUCTION

Curcuma longa (turmeric) is a rhizomatous herbaceous perennial plant of the ginger family. The popular spice curry powder is isolated from the root of the plant to provide the universally distinctive flavor and yellow color. The primary reactive ingredient refined from turmeric is curcumin (1), a poorly soluble yellow-orange powder. The compound has been studied extensively as a therapeutic agent for its anti-inflammatory, antitumor, and antiangiogenesis properties in vitro and in cell culture. However, its remarkable pleiotropic properties span a much wider range. In vivo, curcumin’s applications are unfortunately limited by its low oral bioavailability. Nonetheless, the substance is or has been the subject of clinical trials for the treatment of colon cancer, advanced pancreatic cancer, metastatic breast cancer, inflammatory bowel disease, and cognitive impairment, among over 70 others.

In attempts to improve the solubility, bioavailability, and stability characteristics displayed by curcumin, we previously prepared a panel of curcumin analogues in which both the central and terminal sectors of the molecule were modified to give series 2, as illustrated in Figure 1. Namely, the central keto-enol moiety of 1 was replaced by a monocarbonyl group embedded in a heterocyclic six-membered ring conjugated with the flanking C=C bonds. The terminal oxygenated aromatic rings in 1 were exchanged for fluorophenyl and pyridine moieties, among others. A representative set of analogues are portrayed by structures 3−7. The conjugated curcumin mimics express Michael acceptor properties by delivering conjugates indefinitely stable at room temperature when treated with glutathione. Recently, a convenient NMR assay has provided a simple way to detect Michael adducts and their reversibility, although stable conjugates of curcumin are rarely isolated.

Initially, a set of novel curcumin analogues embodied by 2 were subjected to the National Cancer Institute in vitro cell line screen. The compounds exhibited anticancer and antiangiogenic activities in cell culture. Some revealed a high degree of cytotoxicity, while most of the agents inhibited tumor cell growth with potency greater than that of cisplatin, a well-established clinical chemotherapeutic agent. There are many examples of constitutive or overactivated kinases associated with cancer, and a dozen small molecule inhibitors...
kinase blockers have been approved by the FDA for treatment of the disease. Full mapping of the kinome landscape is projected to have a major influence on personalized cancer treatment. The objective of the present work is to survey the action of 4 on a panel of 50 phosphorylating proteins, including serine/threonine, tyrosine, dual and lipid kinases, in an effort to determine how broadly class 2 penetrates the kinome and to learn if there is a measure of selectivity. We follow up with IC50 measurements on the most strongly blocked kinases with five diverse analogues (3–7), the selection of which is discussed below. Mechanism of action and SAR are pursued by both kinetic measurements with the most active analogue (4 on AKT2) and molecular modeling of the analogues in the AKT2 binding site. Finally, we address the questions of selectivity and pleiotropism by comparing sequences of the ATP binding sites for the kinases screened in the panel of 50. The study does not

Figure 1. Curcumin 1 modifications to generate a series of curcumin analogues.

Figure 2. Screening of a 50-kinase panel by curcumin analogue 4 (EF31) by the Z′-LYTE in vitro kinase assay. (a) 4 was screened against the panel in duplicate at 5 μM with ATP concentrations at K_mapp for each individual kinase. Because of experimental error, values above 100% and below 0% are regarded as maximal or no inhibition, respectively: (*) Adapta and (**) kinase cascade assays. See Experimental Section. (b) Types of kinases among the 50 in both number and % of total. (c) Activity ranges: hits (>80% inhibition), modest activity 40–80% inhibition), and low activity (less than 40% inhibition).
include curcumin itself, since fluorescence interference from this parent molecule interferes with the readouts of the kinase assays employed (see Experimental Section). However, a number of separate studies have shown that curcumin mimics 3−7 are routinely more potent than curcumin by 5- to 50-fold under a variety of circumstances: cytotoxicity toward human prostate and breast cancers,23 anticancer and antiangiogenesis effects,15,24,25 impairment of NF-kB nuclear translocation in mouse macrophages,26 TNF-α-induced NF-kB activation in osteoblastic MC3T3 precursors,27 Fanconi anemia pathway inhibition,28 and stabilization of microtubules in cells.29

RESULTS AND DISCUSSION

To direct the project toward an appropriate selection of cancer kinases, we performed a preliminary screen of 3 and 4 against a small panel of kinases. The compounds had no measurable inhibition of receptor tyrosine kinase IGFR, a weak effect on the cytoplasmic tyrosine kinase Src, moderate inhibition of Ser/Thr kinases (IKKβ, RAF1, and MEK1), and a significantly increased blocking action on AKT1 and AKT2. Results from this screen led to the hypothesis that the current series of curcumin analogues are most likely pleiotropic and, as reported for curcumin, inhibit a range of different kinases.

Kinase Panel Screen. To examine trends and to gain broader knowledge concerning the spread of kinases sensitive to curcumin analogues, we subjected the more potent 4 to screening by a panel of kinases at 5 μM. Since IKKβ and AKT1 were known to be inhibited by the compound prior to conducting the screen, they were inserted as positive controls. Compound 4 indeed proves to be a pleiotropic kinase inhibitor. Of the 50 kinases tested in the profile screen (Figure 2), 10 (20% of the kinases tested) were blocked by 4 at >85% and designated as hits. Compound 4 exhibited 40−80% inhibition against 12 kinases, which were categorized as modestly blocked. The other 28 phosphorylating enzymes (56% of the kinases tested) were inhibited by 4 at ≤40% and are likely to be only weakly influenced by the drug, if at all. In sum, 4 appears to be pleiotropic by blocking a range of kinases with varying potency at 5 μM.

With regard to the selectivity of 4 for specific types of kinases, there is a definite trend. Of the top 10 screening hits, eight were Ser/Thr kinases (25% of Ser/Thr kinases tested), one was a dual function kinase (NEK1, 33% of the dual function kinases tested), and one was a Tyr kinase (KDR, 7% of all Tyr kinases tested). Thus, selectivity for Ser/Thr kinases emerges from this screen. Although the percentage of dual function kinases is higher, it likely results from the small sample
size of this kinase class. Examination of the cross section of kinases with “low activity” reveals a somewhat different balance indicating a potential background role for Tyr kinases: five Ser/Thr kinases (10% of all kinases, 15% of Ser/Thr kinases), six Tyr kinases (12% of all kinases, 40% of Tyr kinases), and one dual function kinase (2% of all kinases, 33% of dual function kinases).

In the present study, any kinase eliciting \( \geq 85\% \) inhibition at 5 \( \mu \text{M} \) was evaluated further by determining IC\(_{50}\) values for 3 (EF24), 4 (EF31), 5 (UBS109), 6 (mono-C=CC reduction product of 5) and the sulfur analogue 7 (SEF31). Justification for this choice has its origin in 3, a compound that performed particularly well in the NCI in vitro cell screen.\(^{13}\) It was subsequently shown to induce apoptosis in two different cancer cell lines\(^{21}\) and inhibit activation of the NF-KB pathway by blocking IKK\(\beta\) kinase.\(^{22}\) This behavior has been posited to contribute to the compound’s anti-inflammatory properties. Analogue 4 has also been shown to inhibit inflammation pathways by inhibiting NF-xB binding in mouse RAW264.7 macrophages with a 10-fold greater potency than 3\(^{23}\) and to inhibit the growth of head and neck squamous cell carcinoma xenografts ip.\(^{30}\) The inclusion of 5 was based on the outcome of a successful oral efficacy study in mice bearing a human tumor xenograft,\(^{31}\) while addition of metabolite 6 resulted from its noteworthy but reduced cellular cytotoxicity relative to 5. Both compounds show a modest suppressive effect on osteosclerogenesis.\(^{25}\) Compound 7, a phosphorylation inhibitor of proteins in the Fanconi anemia pathway,\(^{26}\) an analogue that introduces a large heteroatom into the central ring that occasions a significant change in molecular polarity and simultaneously serves as a poor proton acceptor and weak electron donor by comparison with 3–6 (i.e., 2, \( Y = \text{NH} \) and NMe). The selection permits evaluation of the importance of proton donor and acceptor interactions at the binding sites of the target kinases as well as the influence of protonation and molecular size. These points are taken up in the molecular modeling section below.

**Kinase IC\(_{50}\) Values and an Emerging SAR.** Screened enzymes not previously reported as targets for 3–7 are listed in Figure 3 along with IC\(_{50}\) values for each agent (Table 1). As mentioned above, AKT1 and IKK\(\beta\) were added as positive controls. The kinases represent a wide variety of cellular activities: regulation of protein synthesis and cell proliferation (RPS6KB1), energy sensing and sensitization of cancer cells to cisplatin treatment (AMPK), DNA damage repair and maintenance of mitochondrial membrane potential (NEK1), mediation of cellular responses to VEGF (KDR/VEGFR2), and environmental stress relief and assistance of production of certain cytokines (MAPK14/p38\(\alpha\)), cooperation with the latter kinase to expedite nuclear export, gene expression, and cell proliferation (MAPKAPK2), development, cell proliferation, and apoptosis regulation (RAF1, MEK1, and ERK2). Two kinases blocked by 4 (5 \( \mu \text{M,} \geq 85\% \)), but not examined by dose–response, are CHEK1 and PRKCI/1. The former mediates cell cycle arrest in response to DNA irregularities, while the latter, activated by diacylglycerol, phosphorylates a range of cellular proteins and serves as the receptor for phorbol esters, a class of tumor promoters. Figure 4 captures the network of relationships among the 12 most readily blocked kinases portrayed in Figures 2 and 3. A somewhat more elaborate variation provided by IPA\(^{32}\) is provided in the Supporting Information. Given the tightly connected associations, simultaneous inhibition of these enzymes will exert pairwise-protein influence as well as a major network perturbation. As suggested by the multiple actions of the weaker parent curcumin, 4 is clearly a pleiotropic kinase blocker in addition to exhibiting a \( \geq 10\)-fold greater kinase suppression by comparison with curcumin.\(^{24}\) At the same time, on average, the molecule exhibits only modest potency across the range of enzymes (submicromolar to 7 \( \mu \text{M,} \) Table 1), the exceptions being 4 against AKT and p53\(\alpha\) (MAPK14; see kinase cascade sections below). Other analogues furnish a different profile (Figure 3, Table 1), suggesting an opportunity for reduced toxicity coupled to variable pan-kinase outcomes. Not surprisingly, most of these cellular functions are involved in the promotion of cancer and inflammation. Without yet understanding the details linking the wide diversity of biological responses, modest pleiotropic blockade of this set of kinases appears at the heart of the ability of the curcumin analogues to arrest cancer, angiogenesis, and inflammation both in vitro\(^{15}\)

**Table 1. Calculated IC\(_{50}\) Values from Graphs in Figure 3**

<table>
<thead>
<tr>
<th>kinase</th>
<th>IC(_{50}) (( \mu \text{M} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>AKT1 (PKBo)</td>
<td>0.78</td>
</tr>
<tr>
<td>AKT2</td>
<td>0.72</td>
</tr>
<tr>
<td>IKK(\beta) (IKK(\beta))</td>
<td>72</td>
</tr>
<tr>
<td>RPS6K1</td>
<td>20</td>
</tr>
<tr>
<td>AMPK (A1/B1/G1)</td>
<td>46</td>
</tr>
<tr>
<td>RAF1(^a)</td>
<td>24</td>
</tr>
<tr>
<td>MEK1(^b)</td>
<td>12.8</td>
</tr>
<tr>
<td>ERK2(^b)</td>
<td>13</td>
</tr>
<tr>
<td>NEK1</td>
<td>77</td>
</tr>
<tr>
<td>KDR (VEGFR2)</td>
<td>0.77</td>
</tr>
<tr>
<td>MAPK14 (p38(\alpha))</td>
<td>92</td>
</tr>
<tr>
<td>MAPKAPK2</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

\(^a\)Assays for RAF1 and MEK used the kinase cascade method diagrammed in Figure 5 and discussed in the Experimental Section.

\(^b\)The IC\(_{50}\) values for ERK2 resulted from large experimental variability and should be regarded as only semiquantitative.

**Figure 4.** Phosphorylation and other interactions of transcription factors and enzymes leading to cell proliferation, differentiation, and survival. Kinases highlighted in blue have been studied in this work (Figures 2 and 3). Alternative kinase names are as follows: ERK2 = MAPK1 = MAPK2; p38 = MAPK14; MEK1 = MAPK2; COt = MAP3K; RS1K = RPS6K1; AMPK = PRKAA1; IKK\(\beta\) = IK8KB; KDR = VEGFR-2; RPS6KB1 = S6K1; Chek1 = Chk1.
and in vivo.33 A pertinent example is the behavior of NEK1, the only dual function kinase that is significantly inhibited by 4 in the kinase screen (Figure 2). Among other functions, it contributes to maintenance of the mitochondrial membrane potential. Inhibition of NEK1 causes a decrease in phosphorylation of voltage-dependent anion-selective channel protein 1 (VDAC1), which leads to an uncoupling of the mitochondrial membrane potential.34 Later stages in this event are identical to the manner in which 3 induces apoptosis in two cancer cell lines.21

**Blockade of Signal Transduction Pathways.** We have commented on both individual kinase functions and the potential coupling of kinases in subsections of signaling pathways. The dose–response assays performed for the strongly inhibited kinases involve activation of a given kinase, phosphorylation of a peptide substrate, and disruption of a FRET signal upon cleavage of the peptide to provide the assay readout (Z'LYTE in vitro kinase assay; see Experimental Section and Figure 3). For example, in the measurement of inhibition of RAF1, the procedure takes advantage of the cellular mitogen activated protein (MAP) kinase cascade involving sequential phosphorylation and activation of inactive MEK1 and ERK2 followed by ERK2 phosphorylation of the substrate (Figure 5). By examination of individual kinases, it is clear that curcumin analogues 3, 4, 5, and 7 block all three MAP kinases to within a factor of 3 (Table 1 and Figure 3). This leads to a damping of the entire pathway and eliminates dependence on one specific kinase.

Figure 5. Diagram of kinase cascade used in the Z'LYTE kinase assay. The RAF1 assay is a three-tiered cascade initiated with RAF1, which subsequently phosphorylates and activates MEK1 and ERK2, leading to peptide substrate phosphorylation employed to measure reaction progress (see Z'LYTE in vitro kinase assay in Experimental Section).

A second example from the MAP kinase cascade concerns p38α (MAPK14) and its dependence on MAPKAPK2. Labeling p38α (MAPK14) as a hit in the kinase screen is somewhat misleading, since both kinases are pathway-linked in that screen (Figures 2a and 5). Further examination of p38α (MAPK14) inhibition, however, using a direct kinase assay, shows its blockade to be significantly reduced (Figure 3 and Table 1). Thus, identification of p38α (MAPK14) as a hit in the kinase screen is primarily due to the inhibition of MAPKAPK2 downstream of p38α (MAPK14) in the kinase cascade. It is presently unknown whether assay constitution precisely mirrors these actions in a living cell.

These examples highlight drug action against multiple steps in a localized kinase interaction network by a family of readily accessible curcumin analogues. They simultaneously demonstrate the pleiotropic nature of the analogues both within a select segment of a pathway and among kinases in a more diffuse network (Figure 4 and Figure S1). In a similar manner, the parent compound curcumin inhibits cell signaling cascades at multiple nodes. Since these cascades are involved in cell proliferation and apoptosis, curcumin affects the overall process of carcinogenesis35 as do substances 3–5 and 7.

**Mechanism of Kinase Inhibition.** The curcumin mimics can exert their effects as kinase inhibitors by several possible mechanisms: reversible or irreversible, competition with ATP or the peptide substrate, or allosteric modulation. Since a great many such inhibitors function by competing with ATP and occupying the ATP binding pocket, we sought to explore this possibility by analyzing reaction rates at different ATP and inhibitor concentrations. AKT2 and analogue 4 were selected to probe this question as the most potent combination among kinases and blockers (Table 1 and Figure 3). The corresponding Lineweaver–Burk plot (Figure S3 in Supporting Information) reveals a clustering of lines on the Y axis indicative of a competitive inhibition model.

To achieve a more quantitative evaluation, we plotted the data on a Michaelis–Menten graph. By selection of specific models that force shared parameters in the global analysis (\(K_{i} V_{max} K_{i}\) with GraphPad Prism,36 a compromise solution applied to each model spreads the data across all the curves, providing a framework that allows mechanistic distinctions to be made, namely, competitive or mixed models.37

When performing a global analysis that fits the reaction rates to a Michaelis–Menten graph, the optimized value for \(K_{i}\) is shared by all inhibitor concentration curves. In a competitive model, in addition to the shared \(K_{i}\) constraint, all curves must also maintain a common optimized \(V_{max}\). Furthermore, \(K_{i}\) and

![Graph of enzyme kinetics data for 4 and AKT2](dx.doi.org/10.1021/jm4002692)
$V_{\text{max}}$ must also be greater than zero in both models. Execution of the global analysis under the constraints of a competitive model as illustrated by the corresponding Michaelis–Menten graph significantly worsens the fit of the curves to the data (Figure 6a). By contrast, casting the data in the context of a mixed mechanism provides a superior fit for the data (Figure 6b). Comparison of these two models favors the mixed inhibition model with $p < 0.01$. This suggests that competitive reversible inhibition is not the only mechanistic path traveled by 4 in its inhibition of AKT2. The observation is consistent with a recent study reporting a mixed mechanism for the blockade of IKK/β by analogue 3.²²

To measure the quantitative extent of parallel mechanisms and the impact of substrate concentration (ATP) on kinase inhibition, the parameter $\alpha$ must be examined in the global analysis.³⁸ Its value determines the degree to which the binding of inhibitor alters the affinity of the enzyme for its substrate and is always greater than zero. When $\alpha$ is very small (but greater than zero), binding of the inhibitor enhances substrate binding to the enzyme, and the mixed model becomes nearly identical to an uncompetitive model. When $\alpha = 1$, the inhibitor does not alter binding of substrate to the enzyme, and the mixed-model is identical to noncompetitive inhibition. With increasing $\alpha$, the inhibitor increasingly prevents binding of the substrate, and ultimately the mixed model becomes identical to competitive inhibition.

Using global analysis of the Michaelis–Menten graph, we calculate an $\alpha$ of 31 for the inhibitory action of 4 on AKT2. This value of >1 demonstrates that while compound 4 has multiple mechanisms of inhibition, competition with ATP dominates action against this kinase under these experimental conditions. As mentioned, the mixed inhibition model has been reported for 3 and IKK/β by Kasinski et al.,²² although $\alpha$ was not calculated.

It is tempting to hypothesize that the other mode of inhibition may correspond to covalent binding of inhibitor to enzyme as a result of Michael addition of a free cysteine to the electrophilic enone moiety of 4. This is consistent with our previous demonstration that two glutathione (GSH) molecules are able to combine with a single molecule of 3 and 4 rapidly and quantitatively in aqueous medium followed by isolation of the bis-GSH adducts.¹⁶ The same study demonstrated reversibility in water by exchange of GSH between the two compounds and by the equal effectiveness of the parents and their conjugates (3-GSH)₂ and 4-GSH)₂ to serve as cytotoxic agents against MDA-MB-435 human breast cancer cells. This mechanistic option is discussed below from the viewpoint of the explicit interaction between 4 and AKT2 kinase.

**Analysis of IC₅₀ Data by Molecular Modeling.** We have sought to understand the variation of the ligand IC₅₀ values (Table 1) by assuming that they block the kinases primarily by occupying the ATP binding pocket. Both the kinetic measurements described above for 4 and AKT2 and the previously described action of 3 on IKK/β²² support competitive displacement of ATP as the dominant inhibitory mechanism accompanied by a second action we ascribe to covalent coupling between cysteine and the inhibitor enones. Among the kinases sampled, AKT2 exhibits the lowest IC₅₀ values (Table 1). Accordingly, we selected the crystal structure of the corresponding kinase domain (PDB code 3E88) for pose prediction, once adjusted by the Maestro Protein Preparation Wizard. Ligands 3–7 were docked by Glide into the ATP binding site and optimized by MacroModel.

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**Figure 7.** Top predicted curcumin analogue poses from Glide docking of ligands on AKT2: (a) 4 (EF31); (b) 3 (EF24); (c) 5 (UBSI09).
the enantiomers of prostaglandin enones and dienones.40

3 only one electrophilic enone compared to the two present in an equally unfavorable conformation and the incorporation of benzylic pyridine rings into unfavorable pockets, in one case in

Thus, the loss of a key H-bond, the predicted placement of the bonding of the central six-membered ring. Comparison of the Glide SP docking scores for 5 and 6S/6R predicts the former to be more tightly bound by 2.1 and 2.4 kcal/mol, respectively. Thus, the loss of a key H-bond, the predicted placement of the benzylic pyridine rings into unfavorable pockets, in one case in an equally unfavorable conformation and the incorporation of only one electrophilic enone compared to the two present in 3–5 appear to contribute to the significantly reduced activity of the enantiomers of 6 on AKT2, as has been reported for prostaglandin enones and dienones.40

Analogue 7 possesses a sulfur atom in the central ring instead of nitrogen. In the most favorable pose depicted by Figure 9, the hydrogen bond shared between ligand and Thr292 remains, essentially deleting the hydrogen bond interaction with this residue. In addition, as shown in Figure 7c, two hydrogen atoms in 5 are separated by only 2.0 Å (black stipled line), somewhat below the sum of van der Waals radii (2.4 Å) and thereby introducing internal ligand strain energy. The same H···H distance for 4 (2.3 Å) is at the minimum acceptable van der Waals contact. The diminished hydrogen bonding and ligand strain energy can explain the relatively higher IC50 values of 5 relative to 4.

Partially saturated 6 exists as two enantiomers, 6R and 6S. As shown in Figure 8a and Figure 8b, respectively, the left half of each ligand and the equatorial N-methyl orientations are identical to those of 5. While the stereoisomer poses retain the noncovalent interactions with Thr292 and Glu236, the hydrogen bond with Lys181 is lost as observed for 5. Critical new contacts arise, however, because the C=O saturation in 6 requires the CH2-pyridine moiety to be relocated, placing the relatively hydrophobic edge of the pyridine ring into a polar sector of the protein’s glycine-rich loop. Furthermore, in 6R the pendent CH2-pyridine group fits into the pocket only by adopting a near eclipsed conformation with the adjacent C–H bond of the central six-membered ring. Comparison of the Glide SP docking scores for 5 and 6S/6R predicts the former to be more tightly bound by 2.1 and 2.4 kcal/mol, respectively. Thus, the loss of a key H-bond, the predicted placement of the benzylic pyridine rings into unfavorable pockets, in one case in an equally unfavorable conformation and the incorporation of only one electrophilic enone compared to the two present in 3–5 appear to contribute to the significantly reduced activity of the enantiomers of 6 on AKT2, as has been reported for prostaglandin enones and dienones.40

while association between C=O and Lys181 NH increases to 2.7 Å, weakening the H-bond significantly. The NH to S replacement naturally not only eliminates the hydrogen bond with Glu236 but also causes the ligand to retreat as a consequence of the unfavorable electrostatic contact between the bulky electron-pair bearing sulfur atom and Glu236. The lack of two anchoring H-bonds and the electrostatic disconnect are believed to contribute to the increased IC50 values of 7 compared to 4. In sum, a semiquantitative explanation of the various IC50 values for AKT2 inhibition can be developed by analysis of the docking poses.

Since all the curcumin analogues carry an α,β-unsaturated ketone Michael acceptor, covalent bonds might be formed between kinase cysteines and these compounds. Previously, we reported the modification of cysteine-rich thioredoxin 1 by 3 in the presence of GSH.21 Similarly, Yamakoshi and co-workers have labeled the nuclear fusion protein KSRP/FUBP2 with a biotinylated dienone related to 2 and identified the covalently bound cysteine.41 More recently, Taunton and colleagues have outlined a strategy for targeting noncatalytic cysteines in kinases with reversible covalent inhibitors, suggesting that it can be applied generally to this class of enzymes, assuming there is an exposed cysteine near the active site.42,43 Given the thiol reversibility of our analogues,16 this is a credible, albeit tentative, means to understand the mixed-inhibition observed herein. For example, Figure 10 shows that a cysteine (Cys311

![Figure 8. Top predicted curcumin analogue poses for enantiomers of 6: (a) 6R; (b) 6S.](image)

![Figure 9. Top predicted pose for 7 (SEF31) on AKT2.](image)

![Figure 10. Cys311 in the cleft where the substrate binds on AKT2.](image)
Sequence Comparison of Kinase Binding Sites. We have attempted to understand the pleiotropic aspects of 4 by sequence alignment and identity/similarity comparison of the residues around the ATP binding sites for various kinases. The 3D structures of the proteins were aligned using the protein structure alignment tool in the Schrodinger Maestro software package. By employment of the docking pose of 4 in AKT2, key residues within the ATP binding sites were selected for identity comparison with the corresponding residues in other kinases: Ala179, Met229, Glu230, Tyr231, Ala232, Met282, Glu236, Lys181, and Thr292 (Figure 11). The comparison reveals that most of the active kinases (inhibition of >80% in Figure 2) share >55% identity and >75% similarity with these residues in the AKT2 binding environment while AKT1 and RPS6KB1 share >75% identity and >85% similarity, respectively. KDR is an exception to the >55% and 75% rule, i.e., 33% and 67%, respectively. Further examination of the KDR binding site reveals that two cysteines at the binding site (Cys919 and Cys1045 equivalent to the spatial positions for Ala232 and Thr292 in AKT2) adopt an orientation that favors covalent bonding, suggesting that site geometry is an important feature for both the pleiotropic character of the inhibitors and the Ser/Thr selectivity. A 33% range. Accordingly, kinase binding site structure trends with both the Ser/Thr selectivity and the observed pleiotropic behavior.

**CONCLUSIONS**

By examination of 50 kinases, we have identified a family of enzymes that are inhibited by a small panel of curcumin mimetics, nearly a dozen of which are blocked at micromolar or submicromolar concentrations. Two MAP kinase cascade pathways identified from the present kinase inhibition data are at the heart of tumor development (p38α-MAPKAPK2 and RAF1-MEK1-ERK2). Various oncogenic factors activate these pathways and, by this means, relay proliferative, survival, chemoresistance, and angiogenic signals essential to tumor maintenance. The present observations suggest that the pleiotropic nature of the curcumin analogues leads to damping of pathways without dependence on a single kinase for inhibition (Figure 4 and Figure S1).

Enzyme kinetics studies reveal that kinase inhibition by compounds within series 2 operates by multiple mechanisms, with ATP competition appearing to be a major factor. Complementary molecular modeling at the ATP binding site of AKT2 provides a qualitative explanation for ligand activity. Reversible Michael addition to curcumin mimetics by cysteine residues in the kinases is compatible with the observed mixed mechanism kinetics and the flat IC_{50} values for the KDR kinase. Sequence comparisons for residues at the kinase active sites suggest that site geometry is an important feature for both the pleiotropic nature of the curcumin analogues. From the present kinase inhibition data (Figure 11), we surmise that while the ATP binding site side chain constitution is not the full story, it appears to capture a major structural feature behind much of the pleiotropism of 4, although exceptions such as KDR and NEK1 exist. Other important features of the kinases not captured by Glide docking include the plasticity of the DLG motif and the glycine-rich loop. These aspects are under active investigation for the kinases considered here.

The sequence comparisons of Figure 11 likewise address the predominant selectivity for Ser/Thr kinases by the monocarbonyl inhibitors. Of the ≥85% inhibition hits from 4’s interrogation of the kinase panel, the sequence identities for the Ser/Thr kinases (Figure 2, orange color) range from 56% to 100% relative to AKT2 while the KDR (Tyr kinase) and NEK1 (dual function kinase) exceptions fall at 33%. In a similar fashion, the corresponding sequence identities for Ser/Thr kinases that were poorly blocked (<10% inhibition, PIM1, IRAK4, CDK7, ACVR1B, CDK2, EGFR) also fall in the 22–33% range. Accordingly, kinase binding site structure trends with both the Ser/Thr selectivity and the observed pleiotropic behavior.
a highly successful anticancer agent attenuating, among others, the Ras/MapK pathway, has been approved by the FDA to treat 10 different cancers.\textsuperscript{51,52} It serves as a prime example of the phenomenon.\textsuperscript{53,54}

\section*{EXPERIMENTAL SECTION}

Chemicals, Reagents, and Assay Kits. Compounds 3, 15, 4, 24, 5, 25, 6,\textsuperscript{55} and 7\textsuperscript{15} were prepared at Emory University, GA, as previously described. All compounds were recrystallized and judged to be >95\% pure as determined by HPLC (various concentrations of MeOH in 0.1\% aqueous formic acid solution; Agilent Zorbax 50 mm C18 column; 1 mL/min; monitoring at 254 and 340 nm). For kinase profiling, ADP Quest (catalog no. 90-0071) and ATP Gold (catalog no. 90-0099) were obtained from DisoveRx\textsuperscript{56} while the Cross tide peptide substrate (95\% pure) was available from Millipore (catalog no. 12-331).\textsuperscript{56}

Z'-LYTE kinase assay kits were provided by Invitrogen: SU-Thr 6 (catalog no. PV3179), Ser/Thr 05 (catalog no. PV3178), Tyr 02 (catalog no. PV3191), Ser/Thr 03 (catalog no. PV3176), Tyr 01 (catalog no. PV3190), Ser/Thr 07 (catalog no. PV3180), Ser/Thr 23 (catalog no. PV4644), Ser/Thr 04 (catalog no. PV3177), and Ser/Thr 15 (catalog no. PV3799).

Recombinant proteins came from Invitrogen: AKT1 (PKBz) (catalog no. P2999), AKT2 (PKBz) (catalog no.PV3184), IKKB (IKKβ) (catalog no. PV3836), SRC (catalog no. PV3044), RANKL (RANK) (catalog no. PV3805), inactive MAP2K1 (MEK1) (catalog no. 3093), inactive MAPK1 (ERK2) (catalog no. 3314), MAPK1 (ERK2) (catalog no. PV3313), IGF1R (catalog no. PV3250), RPS6KB1 (MAPKAPK2) (catalog no. PV3304), p38z (MAPK14) (catalog no. PV3304), MAPKAPK2, inactive (catalog no. PV3316), MAPKAPK2 (catalog no. PV3317).

Z'-LYTE in Vitro Kinase Assay (Invitrogen). In a 10 \textmu M kinase reaction, the IKK \zeta transfers the \gamma-phosphate of ATP to a single serine/threonine residue in a specific synthetic peptide substrate (2 \textmu M). The peptide is labeled with the two fluorophores coumarin and fluorescein, one at each end of the peptide to make up a FRET pair. In the development reaction, 5 \textmu L of a site-specific protease recognizes and cleaves any nonphosphorylated peptides. Cleavage disrupts FRET between the coumarin and the fluorescein on the peptide. Phosphorylation of the peptide suppresses cleavage by the protease. Uncleaved, phosphorylated peptides maintain the FRET pair. An amount of 5 \textmu L of stop reagent is added to halt the development reaction before the plate is read by an Envision 2102 plate reader from Perkin-Elmer. During detection, a ratemeter readout of the donor emission over the acceptor emission quantifies reaction progress. The ratio is low if the peptide is phosphorylated, and the ratio is high if the peptide is nonphosphorylated. Percent phosphorylation was calculated using controls, and each compound concentration was run in triplicate. Results were graphed using GraphPad Prism, and IC\textsubscript{50} values were calculated using nonlinear regression.

Fluorescence Interference. Curcumin could not be compared in the Z'-LYTE assay because of fluorescence interference from curcumin in the readouts of the assays used. For instance, the Z'-LYTE assay uses excitation at 400 nm and measures fluorescence of the fluorophores coumarin and fluorescein at 445 and 520 nm respectively. Curcumin, depending on solvent, absorbs light from 350 to 475 nm with maximal absorption around 425 nm. It then fluoresces in the 450–700 nm range\textsuperscript{59} using the 355 nm excitation to measure fluorescence. This would be sufficient to disrupt all aspects of the readout from absorbing the excitation light to absorbing the fluoresced light from coumarin, as well as curcumin fluorescing at wavelengths that are similar to those of fluorescein. These effects were observed when the "test compound fluorescence interference" control was run according the Z'-LYTE assay protocol. The curcumin analogues, however, did not show any fluorescence interference at concentrations up to 50 \textmu M.

Cascade Reaction Assay. Some of the assays require a cascade reaction in which inactivated kinase is activated by an upstream kinase. Two cascades were important for our studies: the RAF1-MEK1-ERK2 cascade and the p38z (MAPK14)-MAPKAPK2 cascade. A direct assay was used for p38z (MAPK14) when generating its nine-point IC\textsubscript{50} curve.

RAF1 (cRAF) Kinase Cascade. The final 10 \textmu L kinase mixture consists of 0.001–0.005 ng of RAF1 (cRAF), 10 ng of inactive MAP2K1 (MEK1), and 100 ng of inactive MAPK1 (ERK2).

MAPK1 (MEK1) Kinase Cascade. The final 10 \textmu L of kinase mixture consists of 1.0–4.0 ng of MAP2K1 (MEK1) and 105 ng of inactive MAPK1 (ERK2).

p38z (MAPK14) Kinase Cascade. The final 10 \textmu L of kinase mixture consists of 0.01–0.02 ng of p38z (MAPK14) and 5 \textmu g of inactive MAPKAPK2.

Kinase Profile Screen. Compound 4 was tested against a panel of kinases at 5 \textmu M. Each data point was measured in duplicate using Z'-LYTE substrates with two control wells. One control well contains a protease control to determine if the compound interferes with the development reaction. The second well encloses a fluorescence control to ascertain if the compound interferes with the fluorescein reading of the FRET pair.

Adapta Assay. After addition of inhibitor and all other kinase reaction components, the reaction was incubated for 1 h. During this period, the kinase reaction produces phosphorylated peptide and ADP. A detection solution of europium-labeled anti-ADP antibodies, a labeled ADP tracer, and EDTA to stop kinase reaction were added. ADP formed by the kinase reaction will displace the ADP tracer from the antibody, resulting in a TR-FRET signal decrease. When an inhibitor is used, the amount of ADP formed by the kinase reaction is reduced, and the resulting intact antibody–tracer interaction results in a high TR-FRET signal. By comparison of this to a control, the amount of inhibition can be calculated.

ADP Quest. Kinase assays for the enzyme kinetics were accomplished with ADP Quest assays. By use of purified recombinant AKT2 from Invitrogen (PV3184) and the Cross tide substrate from Millipore (catalog no. 12-331), kinase reactions were run from 10 to 90 min using fresh samples of varying ATP concentrations. When the Cross tide substrate molecule is phosphorylated, an ADP molecule is generated. The ADP Quest procedure measures total ADP by an enzyme coupled reaction that causes a fluorescent signal. The latter is compared with a known control.

Michaelis–Menten Analysis of Enzyme Kinetics. Analysis of reaction velocities was performed by first determining reaction rates using the ADP quest assay. The reaction rates were measured by following ADP production over time. The reaction velocities were graphed on a Lineweaver–Burk plot and two Michaelis–Menten plots with appropriate constraints for the model of type represented. Calculations were performed with GraphPad Prism 5.\textsuperscript{30}

Molecular Modeling. Docking. The structures of compounds 3, 4, 5, 6, and 7 were drawn in 2D with ChemDraw and then submitted to Ligprep in Maestro 9.1 to obtain 3D structures. Protein complexes were processed by the Protein Preparation Wizard in Maestro followed by removal of the ligands. The receptor grid was generated at the ATP binding sites. Ligand-docking was accomplished with SP precision (Maestro 9.0) for flexible docking of the ligands.\textsuperscript{60,61} The side chain of Lys181 was adjusted to accommodate an H-bond interaction with C\equiv O of the ligand after docking. The PDB codes used in the analysis were as follow: AKT2, 3E88; AKT1, 3MVH; IKKB, 3QAD; NEK1,49B9; RPS6KB1, 3A60; AMPK, 3AQY; PRKCB1, 2OIE; CHEK1, 2YEX; KDR, 2XIR; MAPKAPK2, 3W2M; PIM1, 3QF9; IRAK4, 2OIC; CDK7, 1UA2; KIT, 3GOE; ACVR1B, 1RW8; CDK2, 2XMY; EGFR, 3BE1; PLK1, 3THB; ERBB2, 3PP0.

Generation of a Kinase Interaction Network. A network of the 10 most inhibited kinases by 4 (\textgtrless 85\% inhibition at 5 \textmu M, Figure 2a) was generated using IPA with direct connections (Ingenuity Systems, Inc.).\textsuperscript{26}
IKK/IkB, kinase; IkB, inhibitor of NF-kB; AKT, protein kinase B; ATP, adenosine triphosphate; HT-29, human colon adenocarcinoma; mTOR, mammalian target of rapamycin; NF-kB, nuclear factor κ B; RAW264.7, mouse macrophage-like cell line; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; BCR, breakpoint cluster region; ABL, Abelson leukemia oncogene cellular homologue; Src, Rous sarcoma oncogene cellular homologue; RAF, rapidly accelerated fibrosarcoma; MEK, MAPK/Erk kinase; IGFRI, insulin-like growth factor receptor; Ser/Thr, serine/threonine; Tyr, tyrosine; Cys, cysteine; KDR, kinase domain receptor; RPS6K1, ribosomal protein S6 kinase 1; AMPK, adenosine monophosphate-activated protein kinase; NEK1, never in mitosis gene A-related kinase 1; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor-2; MAPK, mitogen-activated protein kinase; MAPKAP, mitogen-activated protein kinase activated protein kinase; ERK, extracellular signal-regulated kinase; SAR, structure–activity relationship; PRKCI, protein kinase C; p38, stress-activated protein kinase; COT, cancer osaka thyroid; RSK1, ribosomal S6 kinase 1; S6K1, S6 kinase 1; IKKβ, B kinase β; Chk1, checkpoint kinase; Chkl, checkpoint kinase; VDAC1, voltage-dependent anion-selective channel protein 1; FRET, fluorescence resonance energy transfer; PDB, Protein Data Bank

REFERENCES

(22) Kothari, V.; Wei, I.; Shanker, S.; Kalyana-Sundaram, S.; Wang, L.; Ma, L. W.; Vats, P.; Grasso, C. S.; Robinson, D. R.; Wu, Y. M.; Cao, X.; Simeone, D. M.; Chinnaiyan, A. M.; Kumar-Sinha, C. Outlier


(30) Compound 3 has also been shown to exhibit reversibility by the NMR method of Appendino et al. Moore, T.; Snyder, J. P. Unpublished results.


