Thio- and Oxoflavopiridols, Cyclin-Dependent Kinase 1-Selective Inhibitors: Synthesis and Biological Effects

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Flavopiridol analogues, thio- and oxoflavopiridols which contain a sulfur (16) or oxygen (18) atom linker between a chromone ring and the hydrophobic side chain, are selective cyclin-dependent kinase 1 (CDK1) inhibitors with an IC50 of 110 and 130 nM. These analogues were prepared from key intermediate 7 by substituting the ethyl sulfoxide. Enantio pure intermediate piperidone 10 was obtained from the racemic piperidone 8 via a very efficient “dynamic kinetic resolution” in 76% yield. Hydrophobic side chains such as chlorophenyl or tert-butyl produced potent CDK1 inhibitory activity, while hydrophilic side chains such as pyrimidine or aniline caused a severe reduction in CDK inhibitory activity. These analogues are competitive inhibitors with respect to ATP, and therefore activity was dependent upon the CDK subunit without being affected by the cyclin subunit or protein substrate. Thio- and oxoflavopiridols 16 and 18 are not only selective within the CDK family but also discriminated between unrelated serine/threonine and tyrosine protein kinases. CDK1 selective thio- and oxoflavopiridol analogues inhibit the colony-forming ability of multiple human tumor cell lines and possess a unique antiproliferative profile in comparison to flavopiridol.

Introduction

The cyclin-dependent kinases (CDKs) are serine/threonine protein kinases which are the driving force behind the cell cycle and cell proliferation.1,2 CDKs are multisubunit enzymes composed of at least a catalytic subunit (CDK) and a regulatory subunit (cyclin). To date at least 10 CDKs and 15 cyclins have been identified. Once activated, CDKs phosphorylate and modulate the activity of a variety of cellular proteins. These proteins can be categorized as tumor suppressors (e.g., RB, p53), transcription factors (e.g., E2F-DP, RNA pol II), replication factors (replication protein A), and organizational factors that influence cellular and chromatin structures (e.g., histone H1, lamin A, MAP4).3 Individual CDKs perform distinct roles in cell cycle progression and can be classified as either G1, S, or G2/M phase enzymes.1 The kinase activity of CDK complexes is tightly regulated and cell cycle-dependent. This regulation occurs at the level of cyclin gene transcription, cyclin protein degradation, and posttranslational modification of the CDK subunit. In addition, two families of CDK inhibitory proteins have been identified: the KIP proteins p21, p27, and p57 and the INK4 proteins p15, p16, p18, and p19.4 Aberrations in each of these regulatory pathways have been found in a large percentage of human tumors including melanoma, lymphoma, and carcinomas of the breast, prostate, colon, non-small-cell lung, ovarian, pancreatic, and squamous cell.5 As a result, there is considerable interest in restoring normal cell cycle control in tumor cells by targeting CDKs or CDK-associated molecules.5

Toward this end, several ATP-competitive small organic molecules have been reported in the literature as CDK inhibitors. Flavopiridol (1),7 butyrolactone (2),8 olomoucine (3),9 and kenpaullone (4)10 and subsequently several analogues11 of olomoucine and flavopiridol have been reported. While butyrolactone (2) and olomoucine (3) inhibit both CDK1 and CDK2 without having appreciable CDK4 inhibitory activity, flavopiridol (1) is known to have a broad spectrum of CDK inhibitory activity against CDK1, -2, and -4 and is significantly less active against unrelated kinases (Table 3).
it an interesting target for further analogue synthesis. We set out to prepare analogues which would be more selective within the CDK family and possess a unique spectrum of antitumor activity. Herein, we report a series of thio- and oxoflavopiridols that are CDK1-selective inhibitors of the prototype 5 where the piperidylchromone ring is coupled to the hydrophobic R group via a sulfur or oxygen atom linker.

We originally envisioned that thioether 6 or alkyl sulfoxide intermediate 7 would serve as a key intermediate. Replacement of the thioalkyl or alkyl sulfoxide group with the appropriate nucleophiles would expedite analogue synthesis.12 Interestingly, the thio and o xo analogues (Nu = SR or OR) demonstrated CDK1-selective inhibitory activities and displayed unique biological properties, presumably due to an altered selectivity profile. The synthesis, structure–activity relationships (SARs), and biological properties of these compounds are described below.

Chemistry

The key chiral intermediates for the analogue synthesis, thioether 6 and sulfoxide 7, were prepared from chiral piperidone 10 which was obtained from racemic piperidone 812 via a very efficient “dynamic kinetic resolution”14 (Scheme 1). Heating racemic piperidone 8 in the presence of dibenzyl-o-tartaric acid in methanol provides optically pure piperidone 10 in 76% yield after removing dibenzyl-o-tartaric acid. Desired enantiomeric salt 9 was quite insoluble in methanol, while the opposite enantiomeric salt was soluble. There is a difference not only in solubility between these two diastereomeric salts but also in thermodynamic stability. The relative ratio of the two diastereomeric salts existing in hot methanol solution (not in the precipitates) was ca. 4:1 in favor of the desired enantiomeric salt 9 as determined by chiral HPLC column. We believe that a facile in situ epimerization of the C-4 chiral center under the reaction conditions, the thermodynamic stability difference between the two diastereomeric salts in favor of the desired 4R-enantiomer of piperidone 9, and a large solubility difference between the two diastereomeric salts in favor of the desired 4R-enantiomer of piperidone 9, and a large solubility difference between the two diastereomeric salts in methanol enable this “dynamic kinetic resolution” to be very efficient. Enantio pure piperidone 10 was prepared on 100-g scale by this process. Reduction of piperidone 10 using Dibal-H provided the mixture of cis-alcohol 11 (56% yield) and trans-alcohol 12 (13% yield) after purification.

Acylation, which produced concomitant demethylation, followed by treatment of 13 with carbon disulfide produced thiol 14 (Scheme 2). Depending on the nucleophiles, either thioether 6, obtained by the ethylation of thiol 14, or its diastereomeric mixture of sulfoxide 7 was used. Sulfoxide 7 generally led to the higher yields of the product under milder reaction conditions. Demethylation of thioether 15 produced the desired thioflavopiridol 16. Analogues shown in Table 1 (22–26) were prepared in a similar manner using racemic intermediate thioethyl 19 or sulfoxide 20 (Scheme 3).

Results and Discussion

The biological activities of flavopiridol and thio-, oxo-, and other flavopiridol analogues were compared in a series of protein kinase and clonogenic (colony-formation) assays. As illustrated in Table 1, flavopiridol (1) is a potent, broad-spectrum inhibitor of CDKs. Ana-
logues containing a sulfur (16) or oxygen (18) atom linker retain significant potency against CDK1 and gain selectivity within the family of CDKs (Table 1). Thio- and oxoflavopiridols 16 and 18 exhibit selectivity over CDK2 by ~20-fold and 50–150-fold over CDK4, respectively. These compounds showed competitive CDK1 inhibition with respect to ATP, and their selectivities were dependent upon the CDK subunit without being affected by the cyclin subunit (Table 2) or protein substrate (either histone H1 or Rb, data not shown). Thus far the tert-butyl analogue 23 is the most potent CDK1 inhibitor of the sulfur-linked series with IC_{50} = 80 nM as a racemic mixture. This compound also retains significant selectivity within the CDK family. In general, hydrophobic substituents with the sulfur or oxygen atom linker exhibit more potent CDK1/cyclin B inhibitory activity compared to the less hydrophobic analogues, and an aromatic ring is not required for good potency as seen with the tert-butyl analogue 23. On the other hand, more polar substituents, 24 and 25, significantly reduce activity against CDK1. Insertion of a nitrogen atom linker (25) results in a severe reduction in potency against all CDKs tested. As illustrated in Table 3, thio- and oxoflavopiridols 16 and 18 are significantly more selective for CDK1 than other CDK inhibitors described to date. In addition, each appears to retain the excellent selectivity profile of flavopiridol (1) when tested against unrelated serine/threonine and tyrosine protein kinases. In fact, the oxygen-linked analogue 18 is less active against PKC in comparison to flavopiridol (1).

The structures of flavopiridol and thioflavopiridol 16 in complex with CDK2 were determined by X-ray crystallography, revealing the molecular basis of inhibition by these molecules. Figure 1a,b shows the crystal structures of flavopiridol and thioflavopiridol 16, respectively, in the active site of CDK2 with a number of residues comprising the site. In an effort to understand the relative CDK1 selectivity of thioflavopiridol 16 over flavopiridol, an analysis of the X-ray ligand–protein contacts, a comparison of the sequences of CDK1, CDK2, and CDK4, and molecular modeling were performed. The contacts of the benzopyran ring and the piperidinyl ring of both flavopiridol and thioflavopiridol 16 with CDK2 in the X-ray structures are found to be similar to those observed by Kim et al. for deschloroflavopiri-
An analysis of the sequence alignment of CDK1, CDK2, and CDK4 shows only subtle differences in the residues that comprise the buried ATP-binding site; thus selectivity is most likely due to interactions other than those deep in the ATP-binding site. An amino acid comparison of the surface-exposed hinge region and an analysis of flavopiridol and thioflavopiridol docked in homology models of CDK1 and CDK4 (built from the X-ray structure of CDK2, results not published) reveal that several differences in this particular area may be playing a role in specificity (aligned sequences with numbering from SWISS-PROT): CDK1(84-89), SDK2(84-89), HQDLKK; CDK4(97-102), DJDLRT.

The aforementioned differences occur in the region where the chlorophenyl ring of thioflavopiridol resides and may help explain the observed selectivity. The CDK1 selectivity of thioflavopiridol compared to flavopiridol is due to a relative decrease in CDK2 binding. The selectivity may result in part from a less-than-optimal interaction of thioflavopiridol with Ile10, a residue conserved in CDK1, CDK2, and CDK4, coupled with a greater number of compensating interactions with CDK1 than with CDK2 or CDK4. The X-ray structure reveals that the chlorine atom of flavopiridol favorably interacts with Ile10 as a van der Waals distance of 2.99 Å to the C\text{ç} of Ile10 would indicate suggesting that this may be one of the reasons that flavopiridol increases kinase inhibition by a factor of 6 over the deschloro analogue. The CDK2 complex with thioflavopiridol the side chain of Ile10 is observed to be shifted only slightly when the complex is superposed by backbone atoms with that of the flavopiridol–CDK2 complex. However, the sulfur linker of thioflavopiridol places the chlorophenyl ring further away from the side chain of Ile10, relative to flavopiridol, and closer to Lys89 which is observed to be within contact distance of thioflavopiridol. The hydrocarbon portion of the side chain of Lys89 is within van der Waals contact of the chlorophenyl ring. In addition, the basic nitrogen of Lys89 adopts a geometry with respect to the chlorophenyl ring suitable for an

\begin{table}
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\begin{tabular}{|c|c|c|c|c|}
\hline
compd & X & R & IC_{50} (nM) & \\
\hline
1, flavopiridol & none & 2-chlorophenyl & 0.03 & 0.17 & 0.10 \\
19 & S & ethyl (±) & 0.46 & 3.93 & 2.06 \\
16 & S & 2-chlorophenyl (−) & 0.11 & 2.10 & 16.2 \\
18 & O & 2-chlorophenyl (−) & 0.13 & 2.11 & 6.15 \\
21 & S & 2-chlorophenyl (+) & 6.10 & 4.40 & >25 \\
22 & S & phenyl (±) & 0.44 & 6.59 & 4.10 \\
23 & S & tert-butyl (±) & 0.08 & 1.07 & 2.07 \\
24 & S & 4,6-dimethylpyrimidine (±) & 6.40 & 40.4 & 82.5 \\
25 & NH & phenyl (±) & 16.3 & >25 & >25 \\
26 & none & N-piperidyl(±) & 2.50 & 9.69 & 3.70 \\
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\end{tabular}
\caption{CDK Activity Data of the Analogue}
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\begin{table}
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\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
compd & X & R & CDK1/Cyc B1 & CDK2/Cyc E & CDK4/Cyc D1 & IC_{50} (nM) & \\
\hline
1, flavopiridol & none & 2-chlorophenyl & 0.03 & 0.17 & 0.10 & 0.03 & 0.05 & 0.17 & 0.07 \\
16 & S & ethyl (±) & 0.46 & 3.93 & 2.06 & 0.11 & 0.16 & 5.9 & 1.5 \\
\hline
\end{tabular}
\caption{Activity Data of CDK Inhibitors Depending on Cyclin Subunit}
\end{table}

numbering from SWISS-PROT). CDK1(84-89), SDK2(84-89), HQDLKK; CDK4(97-102), DJDLRT.

An analysis of the sequence alignment of CDK1, CDK2, and CDK4 shows only subtle differences in the residues that comprise the buried ATP-binding site; thus selectivity is most likely due to interactions other than those deep in the ATP-binding site. An amino acid comparison of the surface-exposed hinge region and an analysis of flavopiridol and thioflavopiridol docked in homology models of CDK1 and CDK4 (built from the X-ray structure of CDK2, results not published) reveal that several differences in this particular area may be playing a role in specificity (aligned sequences with

\begin{table}
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\begin{tabular}{|c|c|c|c|c|c|c|c|}
\hline
compd & CDK1/Cyc B1 & CDK2/Cyc E & CDK4/Cyc D1 & MAP & PKA & PKC & EGFR \\
\hline
1, flavopiridol & 0.03 & 0.17 & 0.10 & 19.0 & >50 & 14.0 & 22.0 \\
16 & 0.11 & 2.10 & 16.2 & >25 & >50 & 16.1 & >50 \\
18 & 0.13 & 2.11 & 6.15 & >25 & >50 & >50 & >50 \\
olomoucine & 7.0 & 7.0 & 1000 & 30 & 2000 & 1000 & 440 \\
butyrolactone & 0.60 & 1.50 & 1000 & 94 & 260 & 160 & 590 \\
\hline
\end{tabular}
\caption{Activity Data of CDK Inhibitors against Other Different Kinases}
\end{table}
aminoo–aromatic interaction. This type of interaction involving Lys89 was also observed in the crystal structure of a CDK2–clomodine complex. In the flavopiridol–CDK2 complex it is observed that Lys89 is in a different position and closer to Glu85 with a distance from the Lys side chain nitrogen to the Glu side chain oxygen of 3.59 Å. Although Lys89 is common to CDK1 and CDK2, sequence differences near this residue, e.g. Met85 (CDK1) vs Glu85 (CDK2), would not only increase the hydrophobic nature of this area but also affect packing preferences, including the conformational orientation of Lys89, such that it is steered toward the chlorophenyl ring of thioflavopiridol. CDK2/CDK4 selectivity of thioflavopiridol may be rationalized in part by the fact that the corresponding residue of Lys89 (CDK2) in CDK4 is Thr, and thus a potential favorable interaction is lost in binding to CDK4.

Thioflavopiridol 16, the most CDK1-selective compound, was further compared to flavopiridol 1 in a clonogenic assay using four human tumor cell lines (Table 4). This assay demonstrated that changing the CDK selectivity profile impacted the antiproliferative activity of these small molecules. Thioflavopiridol 16 was found to be 16- and 58-fold less active than flavopiridol 1 when tested against HCT116 colorectal carcinoma or A2780 ovarian carcinoma cells. However, thioflavopiridol 16 is equally effective at inhibiting colony formation of PC-3 prostate carcinoma or Mia PaCa-2 pancreatic carcinoma cells. These data suggest that a CDK1-selective inhibitor may have a unique spectrum of antitumor activity and perhaps with altered or lesser side effects. Thus, thioflavopiridol 16, a potent and more selective analogue of flavopiridol, may warrant further preclinical evaluation.

Table 4. Clonogenic Assay Data

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<th>compd</th>
<th>IC₅₀ (nM)</th>
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Conclusions

Enantiopure thio- and oxoflavopiridols were prepared via efficient “dynamic kinetic resolution” of intermediate piperidone 8, and representative thio- and oxoflavopiridols 16 and 18 display CDK1 selective inhibitory activity over CDK2 by 20-fold and 50–150-fold over CDK4, respectively. These compounds do not have any appreciable inhibitory activity against other kinds of kinases such as MAP, PKA, PKC, and EGFR. It is postulated that the CDK1 selectivity of the hydrophobic group-substituted analogues containing a sulfur and oxygen linker presented in this paper is due to sequence differences in the solvent-exposed binding pocket. In a clonogenic assay using human tumor cell lines, nonselective CDK inhibitor flavopiridol and CDK1-selective thioflavopiridol 16 displayed a different spectrum of activity depending on the cell lines. We believe that these small CDK1-selective inhibitor molecules provide us with a unique opportunity to further study the biological role of CDK1/cyclin B.

Experimental Section

All new compounds were homogeneous by thin-layer chromatography and reversed-phase HPLC (>99%). Flash chromatography was carried out on E. Merck Kieselgel 60 silica gel (230–400 mesh). Preparative HPLC was run on YMC OD S-10 50 × 500-mm column eluting with a mixture of solvents A and B (starting from 10% solvent B to 100% solvent B over 30 min gradient time; solvent A: 10% MeOH–90% H₂O–0.1% TFA; solvent B: 90% MeOH–10% H₂O–0.1% TFA; flow rate: 84 mL/min; UV: 254 nm). 1H and 13C NMR spectra were obtained on a JEOL CF-270 spectrometer operating at 270 or 67.5 MHz, respectively, and are reported as ppm downfield from an internal tetramethylsilane standard. The abbreviations of qn and sx in 1H NMR refer to quintet and sextet, respectively. Melting points are uncorrected. Tetrahydrofuran (THF) and xylens were dried by distillation from sodium. N,N-Dimethylformamide (DMF) was dried over 4A molecular sieves.

(1R,1S)-1-Methyl-4-(2,4,6-trimethoxyphenyl)-3-piperidinone (10). A mixture of (±)-1-methyl-4-(2,4,6-trimethoxyphenyl)-3-piperidinone (1.60 g, 5.73 mmol) and dibenzoyl-δ-tartaric acid (2.28 g, 315 mmol) in 10 mL of methanol was heated at reflux temperature until it became a homogeneous solution under argon atmosphere and it was cooled to room tempera-
ature. After stirring overnight at room temperature the precipitated solid was filtered, washed with a small amount of methanol to obtain the first crop of (R)-1-methyl-4-(2,4,6-trimethoxyphenyl)-3-piperidinone dibenzoyl-D-tartaric acid salt, 7 (2.65 g). The filtrate solution was concentrated to a volume of ca. 6 mL and was stirred at ambient temperature overnight. The second crop of the chiral salt (0.24 g) was obtained by adding ethyl ether (150 mL) to the mixture and stirring at room temperature overnight and concentrated. The residue was triturated with a small amount of ethyl acetate and the solid was filtered to obtain crude thiol 14 (6.5 g). This crude thiol compound was used directly for the next step without further purification.

To a stirred solution of crude thiol 14 (6.5 g, 21.01 mmol) at 0 °C in anhydrous DMF (75 mL) under argon was added cesium carbonate (35 g). The resulting suspension was stirred at 0 °C for 30 min, then iodoethane (1.75 g) was added. The mixture was stirred at 0 °C for 1 h and then at room temperature for 18 h. The reaction mixture was diluted with water (300 mL) and the product was extracted with dichloromethane (3 × 300 mL). The combined dichloromethane extracts was washed with 10% aqueous lithium chloride, water and brine, dried over anhydrous Na2SO4 and concentrated in vacuo to give crude 6 (12.5 g, 82.6%) as a light yellow solid: mp 111-113 °C; [a]25D +51.0° (MeOH, c 1.0). Optical purity of 99% of this enantiomer was checked by HPLC on OD Chiracel column (250 × 4.6 mm) eluting with 30% 2-propanol in hexane containing 0.1% triethylamine (flow rate: 1.0 mL/min) at 254 nm: 1H NMR (CDCl3) δ 6.28 (s, 2H), 4.01 (m, 1H), 3.92 (s, 3H), 3.88 (s, 6H), 3.58 (m, 1H), 3.13 (m, 1H), 2.97 (m, 1H), 2.61 (m, 1H), 2.51 (s, 3H), 2.48 (m, 1H), 2.12 (m, 1H); 13C NMR (CDCl3) δ 207.0, 151.5, 159.7, 109.7, 92.3, 67.7, 56.9, 56.4, 55.7, 47.2, 44.6, 30.5; MS (ESI) 446 (M + H)+.

(35S,4R)-1-Methyl-4-(2,4,6-trimethoxyphenyl)-3-piperidinol (11). To a solution of 10 (25.0 g, 95.9 mmol) in CH2Cl2 (225 mL) at −78 °C was added dropwise a solution of disobutylaluminum hydride (180 mmol, 180 mL of 1 M solution in CH2Cl2) under argon atmosphere with maintaining the reaction temperature below −65 °C. The reaction mixture was stirred for 3.5 h at −78 °C after the completion of addition. Trifluoroacetic acid (50 mL) was added dropwise to the reaction mixture at −78 °C. After stirring the mixture for 15 min, MeOH (250 mL) was added. The mixture was warmed to room temperature and concentrated to obtain a solid residue, which was triturated with aqueous NaOH solution (2 N, 750 mL) for 15 min. The product was extracted with ethyl acetate (3 × 750 mL). The combined ethyl acetate solution was washed with brine, dried over Na2SO4 and concentrated to give a gum (25.0 g, 99%). This material was dissolved in CH2Cl2 and purified by flash chromatography (EtOAc:MeOH:Et3N/10:0.5:0.2 and 10:1:0.2) to give 3.81 g of pure 6 (50%) as a solid: [α]20D −74.0° (MeOH, c 0.42); 1H NMR (CDCl3) δ 1.43 (t, J = 7.6 Hz, 3H), 1.58 (m, 1H), 2.05 (m, 1H), 2.23 (m, 1H), 2.35 (s, 3H), 3.02 (m, 5H), 3.26 (s, broad, 1H), 3.35 (m, 1H), 3.86 (m, 1H), 3.94 (s, 3H), 3.97 (s, 3H), 6.43 (s, 1H), 6.46 (s, 1H); 13C NMR (CDCl3) δ 157.8, 165.1, 161.2, 159.7, 157.6, 110.3, 109.9, 108.8, 92.5, 69.9, 62.8, 57.1, 56.4, 56.0, 46.4, 38.4, 25.4, 24.5, 14.2; MS (ESI) 380 (M+H)+. Anal. (C22H26NO5·0.25H2O) C, H, N, S.
as a solid: mp 185–187 °C; [α]D25 59.8° (MeOH, c 0.41); 1H NMR (CDCl3) δ 1.54 (m, 1H), 2.33 (m, 2H), 2.48 (s, 3H), 3.08 (m, 4H), 3.70 (m, 1H), 3.96 (s, 3H), 3.98 (s, 3H), 4.60 (s, 1H), 6.00 (s, 1H), 6.63 (s, 1H), 7.47 (m, 1H), 7.58 (m, 1H), 7.66 (d, J = 8.2 Hz, 1H), 7.80 (d, J = 6.6 Hz, 1H); 13C NMR (CDCl3) δ 179.3, 166.2, 165.9, 162.4, 160.3, 140.8, 139.1, 134.6, 133.3, 130.9, 131.3, 111.6, 109.8, 95.5, 70.5, 63.8, 58.6, 57.9, 57.3, 48.6, 40.3, 25.7; MS (ESI) 462 (M + H).

(3R,4R)-2-(2-Chlorophenylthio)-5,7-dihydroxy-8-(3-hydroxy-1-methyl-4-piperidinyl)-4H-1-benzopyran-4-one (16). To a stirred solution of 15 (2.6 g, 5.63 mmol) in 1,2-dichloroethane (50 mL) under argon was slowly added boron trifluoride (0.5 mL) and the mixture was neutralized by aqueous NaHCO₃ solution. After the addition was complete, the mixture was stirred at room temperature for 10 min. After the addition was complete, the suspension was heated at 85–90 °C for 4 h. The mixture was cooled and then concentrated in vacuo. To the residue at −30 °C (dry ice–acetonitrile bath) methanol (50 mL) was slowly added and the solution was gradually warmed to room temperature. Sodium bicarbonate (8 g) was added as a solid, stirred for 30 min and the mixture was acidified with trifluoroacetic acid to pH 2.0. The mixture was concentrated in vacuo to give a semisolid product (1.69 g). This material was purified by preparative HPLC to obtain the product as a trifluoroacetic acid salt. The trifluoroacetate salt was dissolved in methanol (70 mL) containing aqueous 1 N hydrochloric acid (15 mL), concentrated in vacuo to a small volume and then lyophilized to give 1.94 g (69%) of the HCl salt of sulfoxide product. This crude diastereomeric mixture was separated, dried over magnesium sulfate and concentrated to obtain the trifluoroacetic acid salt (20).

To a solution of 19 (0.99 g, 2.0 mmol) in CH₂Cl₂ (20 mL) containing TFA (0.8 mL) at 0 °C was added m-chloroperbenzoic acid (0.576 g, 50–60% contents) as a solid with stirring. The mixture was stirred at 0 °C for 2 h and then quenched by adding dimethyl sulfide (0.15 mL). The mixture was stirred for 5 min and concentrated in vacuo. To the residue was added ethyl ether (150 mL) and the mixture was stirred at room temperature for 30 min. The precipitated solid was filtered and dried to obtain the trifluoroacetic acid salt of sulfone (21 mg, 60%), which was contaminated by a very small amount of overoxidized sulfone product. This crude diastereomeric mixture was used for the next reaction without any further purification: 1H NMR (CDCl3) δ 6.66 and 6.62 (s, ea, 1H), 6.34 and 6.32 (s, ea, 1H), 4.21 and 4.15 (m, 1H), 3.66–3.04 (m, 8H), 2.91 (s, 3H), 1.88 (m, 1H), 1.32 and 1.26 (t, ea, J = 7.3 Hz, 3H); 13C NMR (CDCl3) δ 182.3 and 182.2, 171.0, 169.8, 165.0, 164.7, 162.8, 156.4 and 158.1, 110.4, 106.9, 107.6 and 106.5, 106.2 and 101.4, 100.9, 67.6, 67.7, 61.9 and 61.8, 56.7, 47.4, 46.6, 44.4 and 43.7, 37.5 and 37.3, 23.5, 6.1, 5.2.

(3R,4R)-2-(Ethylsulfonyl)-8-(3-hydroxy-1-methyl-4-piperidinyl)-5,7-dihydroxy-4H-1-benzopyran-4-one, Trifluoroacetic Acid Salt (20). To a solution of 19 (50 mg, 0.1 mmol), thiophenol and NaHCO₃ (100 mg) in DMF (0.5 mL) was stirred at 100 °C for 2 h, cooled to room temperature and the solid was filtered off. The filtrate solution was purified by preparative HPLC to obtain the trifluoroacetic acid salt of sulfone (21) as a solid: mp 62–74 °C; 1H NMR (CDCl3) δ 7.73–7.59 (m, 5H), 6.24 (d, J = 10.8 Hz, 1H), 5.86 (s, 1H), 3.13 (t, J = 6.6 Hz, 1H), 2.85 (m, 6H), 2.28 (s, 3H), 1.82–2.05 (m, 1H), 1.48–1.60 (m, 1H); 13C NMR (CDCl3) δ 179.4, 164.1, 162.2, 159.9, 155.1, 147.8, 131.8, 128.7, 128.1, 127.3, 123.4, 111.0, 108.2, 93.2, 91.1, 70.1, 62.8, 57.1, 56.7, 56.3, 46.7, 46.4, 38.4, 24.8; MS (ESI) 446 (M + H)⁶.

(3R,4R)-2-(2-Chlorophenylthio)-5,7-dihydroxy-8-(3-hydroxy-1-methyl-4-piperidinyl)-4H-1-benzopyran-4-one (17). A mixture of 2-chlorophenol (1.0 mL, 9.65 mmol) and potassium tert-butoxide (1.2 g, 10.69 mmol) in anhydrous THF (12 mL) was stirred for 10 min at room temperature, then cooled in an ice bath and sulfide 7 (1.15 g, 2.26 mmol) was added. The reaction mixture was stirred at 0–5 °C for 1.5 h. After adding acetic acid (0.25 mL) to the mixture it was directly loaded onto a silica gel column and eluted with ethyl acetate followed by EtOAc:MeOH:Et₃N (100:20:2) to obtain after concentration a product containing trifluoroacetic acid and triethylamine. This material was mixed with chloroform (60 mL) and aqueous NaHCO₃ solution, the organic layer was washed with sodium chloride (10 mL), then dried over sodium sulfate and concentrated to obtain the trifluoroacetic acid salt of (C₁₇H₂₁NO₅S)₂C₆H₅CF₃COOH) C, H, N. Anal. (C₁₇H₂₁NO₅S)₂C₆H₅CF₃COOH) C, H, N. 60% contents)
Table 5. Summary of the Crystallographic Data. Collection, Reduction, and Refinement

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Kinase reactions consisted of 5 ng of baculovirus expressed GST-CDK2/cyclin E (human), complex, 0.5 μg GST-RB fusion protein (amino acids 776–928 of retinoblastoma protein), 0.2 μM [γ-32P]ATP, 25 μM ATP in 50 μL kinase buffer (50 mM Hepes, pH 8.0, 10 mM MgCl2, 1 mM EGTA, 2 mM DTT). Reactions were incubated for 45 min at 30 °C and stopped by the addition of cold trichloroacetic acid (TCA) to a final concentration 15%. TCA precipitates were collected onto GF/C unifilter plates (Packard Instrument Co., Meriden, CT) using a Filtermate universal harvester (Packard Instrument Co., Meriden, CT) and the filters were quantitated using a TopCount 96-well liquid scintillation counter (Packard Instrument Co., Meriden, CT). Dose–response curves were generated to determine the concentration required to inhibit 50% of kinase activity (IC50). Compounds were dissolved at 10 mM in dimethyl formamide (DMF) and evaluated at six concentrations, each in triplicate. The final concentration of DMF in the assay equaled 2%. IC50 values were derived by nonlinear regression analysis and have a coefficient of variance (SD/mean, n = 6) = 14%.

CDK4/Cyclin D1 Kinase Assay. Kinase reactions consisted of 150 ng of baculovirus expressed GST-CDK4/cyclin D1 (human), 280 ng of Stag-cyclin D1, 0.5 μg GST-RB fusion protein (amino acids 776–928 of retinoblastoma protein), 0.2 μM [γ-32P]ATP, 25 μM ATP in 50 μL kinase buffer (50 mM Hepes, pH 8.0, 10 mM MgCl2, 1 mM EGTA, 2 mM DTT). Reactions were incubated for 1 h at 30 °C and stopped by the addition of cold trichloroacetic acid (TCA) to a final concentration 15%. TCA precipitates were collected onto GF/C unifilter plates (Packard Instrument Co., Meriden, CT) using a Filtermate universal harvester (Packard Instrument Co., Meriden, CT) and the filters were quantitated using a TopCount 96-well liquid scintillation counter (Packard Instrument Co., Meriden, CT). Dose–response curves were generated to determine the concentration required inhibiting 50% of kinase activity (IC50). Compounds were dissolved at 10 mM in dimethyl formamide (DMF) and evaluated at six concentrations, each in triplicate. The final concentration of DMF in the assay equaled 2%. IC50 values were derived by nonlinear regression analysis and have a coefficient of variance (SD/mean, n = 6) = 18%.

Kinase assays for PKA and EGFR were carried out using a TCA precipitation procedure identical to that described earlier. PKA (2 units) from bovine heart (Sigma Chemical Co.) was reacted with 500 ng of GST-TK fusion protein substrate (generated using pGEX-2TK expression construct, Pharmacia) for 1 h at 30 °C in 50 mM Tris pH 7.5, 10 mM MgCl2 and 25 μM [γ-32P]ATP. EGFR (50 ng catalytic subunit domain expressed in baculovirus infected S9 cells as a GST-fusion protein) was reacted with 1 μg poly(Glu-Tyr) for 1 h at 30 °C in 50 mM Tris pH 7.5, 10 mM MgCl2, 2 mM MnCl2 and 25 μM [γ-32P]ATP.

The MAP kinase and PKC reactions were quantitated by spotting onto p81 phosphocellulose as described for the PKC assay system from Gibco BRL. MAP kinase p42, of xenopus origin (Santa Cruz Biotechnology Inc.), was reacted with 1 μM substrate peptide (APRTPGGR) for 1 h at 30 °C in 50 mM Tris pH 8.0, 10 mM MgCl2, 0.5 mM EGTA, 1 mM DTT and 25 μM [γ-32P]ATP. PKC was assayed using a 1 μM assay from Gibco BRL at an ATP concentration of 20 μM. PKC (mixture of α, β and γ isoforms from rat brain) was purchased from Upstate Biotechnology.

Clonogenic Assay. Colony growth inhibition was measured for four human tumor cell lines (HCT116 colorectal carcinoma, A2780 ovarian carcinoma, A549 lung carcinoma and Mia PaCa-2 pancreatic carcinoma) using a standard clonogenic...
assay. Briefly, 200 cells/well were seeded into 6-well tissue culture plates (Falcon, Franklin Lakes, NJ) and allowed to attach for 18 h. Assay medium consisted of either MCcoy's 5a, RPMI-1640, Ham's F12K or DMEM plus 10% fetal bovine serum for the HCT116, A2780, A549 and Mia PaCa-2 cell lines, respectively. Cells were then treated in duplicate with a six concentration dose–response curve. The maximum concentration of DMF never exceeded 0.25%. Compound was replaced with fresh medium every third day. Colony number was scored on day 10 using a BioTran III colony counter (New Brunswick Scientific Co., Edison, NJ). The compound concentration required to inhibit 50% of colony formation (IC50) was determined by nonlinear regression analysis. The coefficient of variance (SD/mean, n = 3) = 30%.

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References: