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LETTER

Synthesis and Biological Activity of Novel Pyranopyrones Derived from Engineered Aromatic Polyketides

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ABSTRACT The 4-hydroxy-2-pyrone moiety is commonly observed in polyketides generated *via* biosynthetic engineering of type II polyketide synthases. To explore the synthetic utility of these 2-pyrones, four engineered polyketides (mutactin, SEK4, SEK15, and SEK15b) were isolated from appropriate derivatives of *Streptomyces coelicolor* CH999. As a test case, we prepared nine novel pyranopyrones through condensation reactions with either citral, 1-cyclohexenecarboxaldehyde, or *S*-perillaldehyde. Synthetic tricyclic pyranopyrones with simple aromatic substituents are known to possess anticancer properties. We therefore investigated whether pyranopyrone derivatives of aromatic polyketides exhibited bioactivity in a panel of three cancer cell lines. Pyranopyrones generated from SEK4 had activity comparable to that of H10, a pyranopyrone with a 3-pyridyl substituent, whereas other analogues were less active. These results suggest that the diverse library of carbo- and heterocycles available through the genetic engineering of type II polyketide synthases can serve as useful building blocks to generate unique bioactive molecules.

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Aromatic polyketides, a group of natural products that includes the clinically used tetracycline and doxorubicin, are biosynthesized through iterative decarboxylative condensation of malonyl-coenzyme A (CoA) to yield an intermediate polyketide chain of controlled length that is subsequently tailored by downstream polyketide synthase (PKS) enzymes to yield the observed natural products. This intermediate polyketide chain is generated by an enzyme complex known as a minimal PKS, which consists of a heterodimeric ketosynthase-chain length factor (KS-CLF) responsible for the initial loading and condensation of the malonyl units, an acyl-carrier protein (ACP) that transfers malonyl units to the KS-CLF, and a malonyl-CoA:ACP acyl transferase that loads malonyl-CoA onto the ACP (*1*). Aromatic polyketides of varying length are produced by different minimal PKSs. For example, the actinorhodin (*act*) minimal PKS produces an octaketide (C_{16}) (*2*), the tetracenomycin (*tcm*) minimal PKS produces a decaketide (C_{20}) (*3*), and the *Streptomyces coelicolor* spore pigment minimal PKS (*whiE*) when expressed by itself primarily produces undecaketides (C_{22}) or dodecaketides (C_{24}) (*4, 5*).

Over the past 15 years, a wide variety of hetero- and carbocycles have been biosynthesized by *Streptomyces* strains containing engineered expression plasmids encoding for alternative minimal PKSs, as well as auxiliary ketoreductases, aromatases, and/or cyclases. Examples of these include DMAC (**1**) (*2*), mutactin (**2**) (*6*), SEK4 (**3**) (*7*), SEK15 (**4**) (*7*), SEK15b (**5**) (*6*), RM20b (**6**) (*3*), RM80 (**7**) (*8*), and PK8 (**8**) (Figure 1) (*9*). As seen in Figure 1, these compounds have variable structural backbones yet often contain a 4-hydroxy-2-pyrone functionality, which is the cyclization product of the terminal triketide as it is released from the ACP (*10*). These engineered polyketides can therefore be viewed as a structurally diverse library of 2-pyrones, which if capable of being regioselectively functionalized could be used to prepare semisynthetic natural products of medicinal value.

The tricyclic pyranopyrones are a class of anticancer agents that are prepared from the condensation of a 4-hydroxy-2-pyrone with the α,β-unsaturated aldehyde 1-cyclohexenecarboxaldehyde (*11*). This reaction proceeds by a domino Knoevenagel–6π-electrocyclization mechanism in which an amine-activated aldehyde is subjected to nucleophilic attack by the pyrone. β-Elimination of the amine results in the formation of a 1-oxatriene, which undergoes intramolecular cyclization to form a dihydropyran ring (*12*). This reaction has been employed in the synthesis of natural products such as (±)-daurichromenic acid (*13*), as well as in the preparation of simple pyrone derivatives (*11, 12, 14*).

The most investigated member of the tricyclic pyranopyrones, H10 (**9**, Figure 2), has been found to display antitumor biological activity. H10 inhibits growth of the murine leukemia L1210 cell line and the murine EMT-6 mammary cancer cell line with GI_{50}'s of 1.1 and 1.5 μM, respectively (*15, 16*). Further evaluation of H10 has demonstrated that it inhibits tubulin polymerization(IC_{50}

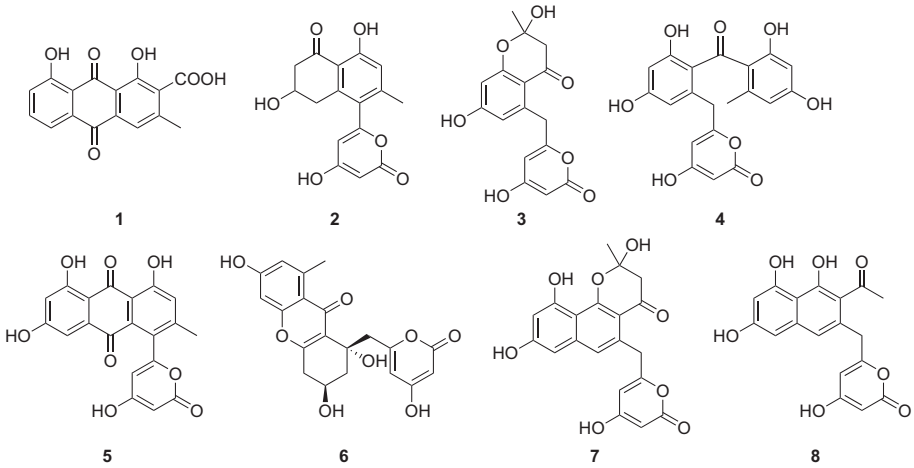

Figure 1. Structures of engineered type II polyketides.

$= 1.5\ \mu M$) and the cellular transport of nucleosides ($IC_{50} = 6\ \mu M$) (*16, 17*). When investigated in an *in vivo* mouse model, the hydrochloride salt of H10 decreased the growth of solid Lewis lung cancer tumors by 67% over 14 d when administered in six 30 mg kg^{-1} doses (qd) on days 1–6 (*17*).

Some tricyclic pyranopyrone analogues of H10 have been prepared, and their effectiveness against L1210 *in vitro* was evaluated. The regioisomer H19 (**10**, Figure 2) is $3\times$ less potent than H10 (*17*). Substituting the 3-pyridyl group with a 3,4-dimethoxyphenyl ring (H5, **11**) results in an order of magnitude loss of potency (*15*). The pyranopyrone analogue H12 (**12**), which lacks an aromatic substituent, had little activity when screened at 50 μM, indicating that the appended aromatic functional group is critical for the activity of these compounds (*15*).

Other tricyclic pyranopyrone analogues prepared with aldehydes, alcohols, aliphatic groups, or adenine had similar or lower activity, leaving H10 as the lead compound for this class of compounds (*17*).

Because the aromatic substituent was shown to be important for the biological activity of these compounds, we wished to utilize engineered aromatic polyketides containing 4-hydroxy-2-pyrone moieties to generate pyranopyrone analogues of considerably greater complexity than previously achieved through synthesis alone. Therefore, four polyketides were chosen in this study that contained diverse aromatic functional groups either fused directly to a 4-hydroxy-2-pyrone or *via* a methylene bridge. The tatralone mutactin (**2**), the hemiacetal SEK4 (**3**), the diphenyl ketone SEK15 (**4**), and the quinone SEK15b (**5**) were utilized for condensation reactions with the α,β-unsaturated aldehydes citral (**13**), 1-cyclohexenecarboxaldehyde (**14**), and *S*-perillaldehyde (**15**). To evaluate their biological activity, the obtained analogues were screened against the L1210 murine leukemia cell line, the K-562 human leukemia cell line, and the HCT-116 human colon tumor cell line in comparison with H10.

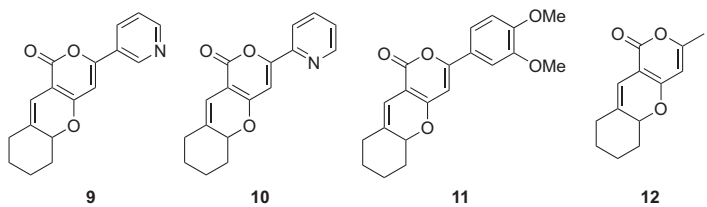

Figure 2. Tricyclic pyranopyrones evaluated as cytotoxic agents.

Synthesis of Analogues. The engineered polyketide 4-hydroxy-2-pyrones contain reactive functionalities such as hemiacetals and phenols not found in synthetic pyrones, and a concern was that condensation reactions with α,β-unsaturated aldehydes would not proceed in a regiospecific manner. Therefore, SEK4 (**3**, Figure 1) was chosen as a trial polyketide (containing both a phenol and a hemiacetal) and citral (**13**, Figure 3) as the trial α,β-unsaturated aldehyde, which was previously shown to proceed in 94% yield when treated with 4-hydroxy-6-methyl-2*H*-pyran-2-one (*14*). At RT in the presence of 1.1 equiv of citral, 0.5 equiv of β-alanine, and anhydrous calcium sulfate in 10:39:1 methanol/ethyl acetate/acetic acid, SEK4 was fully reacted in 2 h as monitored by TLC to yield one major product with a molecular weight of $C_{26}H_{28}O_7$ based on a high-resolution mass measurement (475.1733 $[M + Na]^+$, Δ +0.1 ppm), consistent with the desired product. ^1H and ^{13}C NMR spectra in DMSO-d_6 were compared to the spectra of SEK4. The ^1H NMR chemical shifts at $\delta =$ 6.89 and 10.55 were similar to the shifts of $\delta = 6.90$ and 10.50 found in SEK4 (*7*), indicating that the hemiacetal hydroxyl and the phenol at C-9 were intact. Additional ^1H and ^{13}C peaks were present in the product, suggesting that the anticipated pyranopyrone had formed. Olefinic doublets present in the ^1H NMR spectrum at $\delta = 6.25$ and 5.48 (H-2′ and H-3′, $J = 10$ Hz), two vinyl methyl singlets at 1.59 and 1.49 ppm, and a qua-

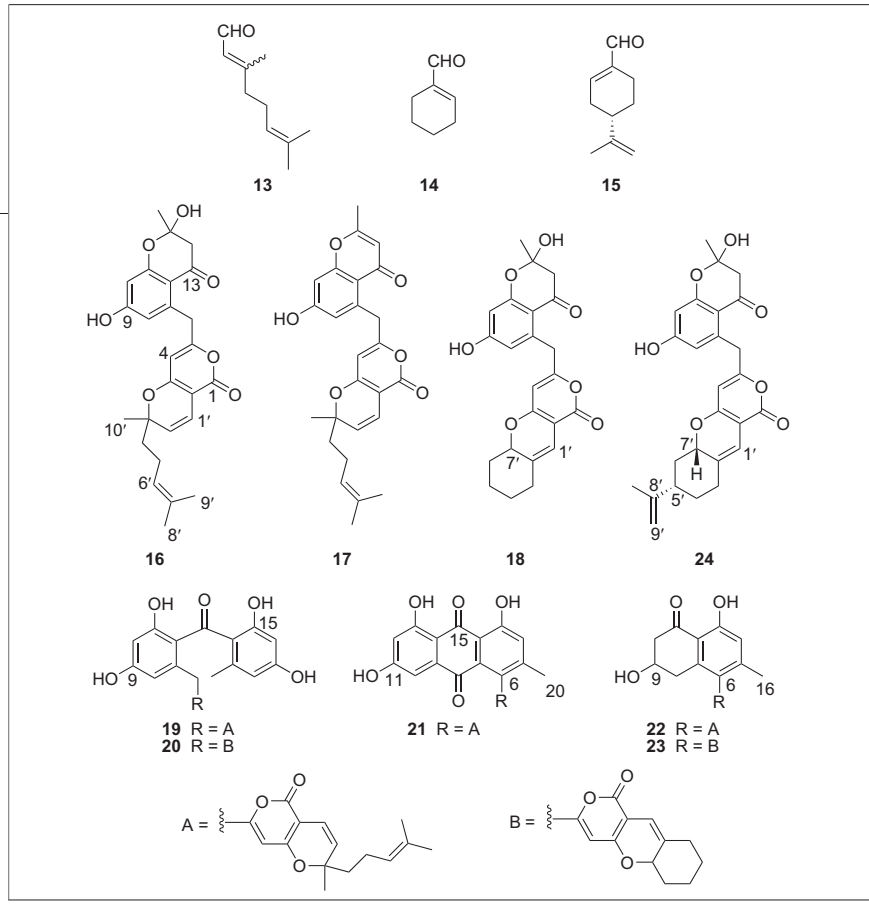

Figure 3. Aldehydes used (13–15) and the pyranopyrones prepared (16–24) in this study.

ternary ^{13}C peak at $\delta = 82.7$ (C-3′) indicating an oxygen subsitituent were all consistent with previously reported citral adducts of 4-hydroxy-2-pyrones (*14*). Heteronuclear single quantum correlation and heteronuclear multiple bond borrelation data allowed assignment of all ^{1}H and ^{13}C signals and confirmed the structure as SEK4/c (**16**). SEK4/c was found to be stable when purified with reverse phase C_{18} HPLC using an acetonitrile/water gradient and could be isolated in 69% yield. As a consequence of using acidic reaction conditions, dehydroSEK4/c (**17**) was also isolated in 6% yield. SEK4 could also be treated with 1-cyclohexenecarboxaldehyde under the same reaction conditions to yield SEK4/cc (**18**) in a lower yield of 34%, although the rate of the reaction was slower (48 h instead of 2 h).

These reaction conditions used for SEK4 were then extended to SEK15, SEK15b, and mutactin. Products for both condensation reactions were obtained for SEK15 and mutactin, but only the citral annulation led to product for SEK15b (**19**–**23**). Two attempts were conducted to react SEK15b with 1-cyclohexenecarboxaldehyde, but both times a mixture of products resulted as found by HPLC analysis. Therefore, it seems likely that the quinone substituent was not stable for 48 h under the conditions used for the 1-cyclohexenecarboxaldehyde reaction.

These reactions were not anticipated to proceed in an enantio- or diastereoselective manner on the basis of previous work (*11, 14*), and consistent with this proposal SEK15b/c (**21**) and SEK15/cc (**20**) had optical rotations equal to that of solvent. Because mutactin (**2**, Figure 1) is a racemate (*18*), a non-diastereoselective reaction would result in four diastereomers that can have different chemical shifts. Multiple ^{1}H and ^{13}C NMR signals were observed for some protons and carbons in mutactin/c (**21**, Figure 3) and mutactin/cc (**23**), indicating the presence of diastereomers in the isolated product and a non-stereoselective course of these condensation reactions under the conditions used. To evaluate whether the observed biological activity of the SEK4 analogues required the presence of racemic mixtures of the pyranopyrone ring system, SEK4 was treated with *S*-(−)-perillaldehyde (**15**) under the same conditions used for the 1-cyclohexenecarboxaldehyde reaction to generate SEK4/p (**24**). The presence of the C-4 functional group of *S*-perillaldehyde causes an efficient asymmetric induction (*11*) leading to the 5′*S*,7′*S*-pyranopyrone as the only product.

Biological Evaluation. All nine pyranopyrones in Figure 3 were evaluated as growth inhibitors of the L1210 murine leukemia cell line, the K-562 human leukemia cell line, and the HCT-116 human colon tumor cell line. The parent 4-hydroxy-2-pyrones were all found to be inactive in all three cell lines when tested at 40 μM concentration. The lead pyranopyrone H10 (**9**, Figure 2) and the topoisomerase II inhibitor etoposide were used as positive controls. The GI_{50}'s for each compound are shown in Table 1. Mutactin/cc (**23**) and SEK15/cc (**20**) were at least an order of magnitude less active in all three cell lines compared to H10, indicating that the tetralone and diphenyl ketone substituents are detrimental to the biological activity of the pyranopyrones. The citral adducts of mutactin and SEK15 also had poor activity. SEK15b/c (**21**) was an order of magnitude less active in the L1210 and HCT-116 cell lines, although it did appear to demonstrate slight (2-fold) selectivity for the K-562 cell line. SEK4/cc (**18**) and SEK4/p (**24**) had low micromolar activity in all three cell lines, similar to H10, indicating that the hemiacetal is an acceptable substituent on the tricyclic pyranopyrone and that a racemic mixture at C-7′ isn't necessary for biological activity. The citral adduct SEK4/c (**16**) also had low micromolar GI_{50} values in all cell lines, indicating that additional variation of the pyranopyrone core is tolerated. Evaluation of dehydroSEK4/c (**17**) revealed that the hemiacetal is critical for the observed biological activity, as only weak activity was observed in the three cell lines. Because SEK4 (**3**, Figure 1) was inactive when screened against all three cell lines at 40 μM concentration, the hemiacetal by itself is not sufficient for the observed biological activity of the SEK4 analogues, an indica-

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TABLE 1. GI_{50} values (μM) for each compound toward the three cancer cell lines

Compound	L1210	K-562	HCT-116
Etoposide	0.14	0.13	0.65
H10 (**9**)	1.6	2.3	3.9
SEK4/c (**16**)	7.9	2.7	3.3
Dehydro-SEK4/c (**17**)	56	71	89
SEK4/cc (**18**)	5.7	3.4	3.7
SEK15/c (**19**)	53	59	62
SEK15/cc (**20**)	57	73	68
SEK15b/c (**21**)	20.6	9.2	27.4
Mutactin/c (**22**)	19.5	55	31.1
Mutactin/cc (**23**)	25.5	17.3	21.4
SEK4/p (**24**)	3.7	2.9	3.3

tion that the pyranopyrone ring system is also important for the observed biological activity.

In summary, aromatic engineered polyketides provide a source of structurally diverse 4-hydroxy-2-pyrones that can be regioselectively functionalized with α,β-unsaturated aldehydes to yield pyranopyrone analogues. Biological evaluation of nine such analogues has revealed that the hemiacetal-containing pyranopyrones have activities similar to that of H10, revealing that there is flexibility in both the aromatic substituent on the pyranopyrone and the architecture of the pyranopyrone itself in the observed *in vitro* cytotoxic activity. Other pyranopyrones generated in this study containing a quinone, diphenyl ketone, or tetralone functionality in general had only weak to moderate activity compared to that of H10. Therefore, these larger and more complex substituents are not well tolerated, supporting previous results that the type of aromatic substituent on the pyranopyrone plays a big role in the observed cytotoxic activity of this family of compounds.

By using the reaction conditions described here, it should be possible to functionalize further structurally diverse polyketide 2-pyrones with a wide variety of α,β-unsaturated aldehydes, as has been published for other synthetic 2-pyrones (*11–13, 19*). Although some engineered polyketides may not be amenable for these condensations, such as SEK15b when reacted with 1-cyclohexencarboxaldehyde, we have shown that other reactive functional groups commonly found on these polyketides, such as phenols and hemiacetals, can be acceptable. To our knowledge, this is the first example of semisynthetic exploitation of a conserved functional group in "unnatural" natural products with the goal of developing new bioactive compounds. Future efforts along these directions promise to yield small molecules that are unlikely to be obtained through biological or synthetic approaches alone.

METHODS

Synthesis of Pyranopyrones. Typical Procedure for the Citral Condensation Reaction with SEK4 as the 2-Pyrone. A solution of SEK4 (**3**, 10 mg, 0.031 mmol) suspended in 2 mL of 10:39:1 methanol/ethyl acetate/glacial acetic acid was dried with anhydrous sodium sulfate, added to a round-bottom flask along with anhydrous calcium sulfate (8 mg) and β-alanine (1.4 mg, 0.016 mmol), and stirred for 10 min. Citral (**13**, 5.2 mg, 0.034 mmol) was added, and the reaction solution was stirred for 2 h at RT. The solution was then centrifuged to remove particulates, and the supernatant was decanted, from which the solvent was removed under reduced pressure. The resulting oil was subjected to HPLC on a C_{18} preparative column using an elution gradient of 50% to 80% CH_3CN/H_2O to yield SEK4/c (**16**, 9.7 mg, 69%) and dehydroSEK4/c (**17**, 0.8 mg, 6%). This reaction was repeated to obtain a total of 3 mg of dehydroSEK4/c for characterization and biological evaluation.

Extension of the above reaction conditions to SEK15 (**4**, 5.0 mg, 0.013 mmol), SEK15b (**5**, 5.0 mg, 0.013 mmol), and mutactin (**2**, 9.6 mg, 0.032 mmol) led to SEK15/c (**19**, 6.6 mg, 98%), SEK15b/c (**21**, 3.7 mg, 55%), and mutactin/c (**22**, 10.2 mg, 73%), respectively. The HPLC gradient for purification of SEK15/c was 40% to 70% CH_3CN/H_2O, for SEK15b it was 50% CH_3CN/H_2O to 100% CH_3CN, and for mutactin/c it was 50% to 80% CH_3CN/H_2O.

Typical Procedure for the 1-Cyclohexenecarboxaldehyde Condensation Reaction with SEK4 as the 2-Pyrone. A solution of SEK4 (**3**, 20 mg, 0.062 mmol) suspended in 4 mL of 10:39:1 methanol/ethyl acetate/glacial acetic acid was dried with anhydrous sodium sulfate, added to a round-bottom flask along with anhydrous calcium sulfate (16 mg) and β-alanine (2.8 mg, 0.032 mmol), and stirred for 10 min. 1-Cyclohexenecarboxaldehyde (**14**, 7.6 mg, 0.068 mmol) was added, and the reaction solution was stirred for 48 h at RT. The solution was then centrifuged to remove particulates, and the supernatant was decanted, from which the solvent was removed under reduced pressure. The resulting oil was subjected to HPLC on a C_{18} preparative column using an elution gradient of 50% to 65% CH_3CN/H_2O to yield SEK4/cc (**18**, 8.7 mg, 34%) and dehydrated SEK4/cc (0.9 mg, 3%).

Extension of the above reaction conditions to SEK15 (**4**, 20.0 mg, 0.052 mmol) and mutactin (**2**, 10 mg, 0.033 mmol) led to SEK15/cc (**20**, 12.2 mg, 49%) and mutactin/cc (**23**, 3.8 mg, 26%), respectively. The HPLC gradient for purification of SEK15/cc was 40% to 65% CH_3CN/H_2O, and for mutactin/cc it was 50% to 65% CH_3CN/H_2O.

Treatment of SEK4 (**3**, 23.0 mg, 0.072 mmol) with *S*-(−)-perillaldehyde (**15**, 11.9 mg, 0.079 mmol) under the same conditions used for the 1-cyclohexenecarboxaldehyde reaction yielded SEK4/p (**24**, 8.0 mg, 25%) and dehydrated SEK4/p (0.4 mg, 1%). The HPLC gradient used for the purification of SEK4/p was 50% to 65% CH_3CN/H_2O.

Cancer Cell Line Screen. Murine L1210 leukemia (ATCC no. CCL-219) cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 media with Glutamax (Gibco) containing 10% heat-inactivated fetal bovine serum (FBS, Hyclone) and 100 U mL^{-1} penicillin/100 μg streptomycin sulfate (PS, Gibco). Human K-562 leukemia (ATCC no. CCL-243) cells were maintained in Iscove's Modified Dulbecco's Medium with Glutamax, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, and 3.024 mg L^{-1} $NaHCO_3$ (Gibco) further amended with 10% FBS and PS. The human colon tumor HCT-116 cells (ATCC no. CCL-247) were maintained in McCoy's 5a medium with L-glutamine (Gibco) adjusted to contain 2.2 g L^{-1} sodium bicarbonate, 10% FBS, and

PS. The L120 and K-562 cell lines were cultured in continuous exponential growth at 37 °C in 5% CO_2/air, and the HCT-116 cells were maintained at 30–90% confluency.

For the growth inhibition assays, all three cell types were resuspended at a concentration of 2.5 $\times$ 10^4 cells mL^{-1} in RPMI 1640 media, including L-glutamine without phenol red (Gibco) amended with 10% FBS and PS. An aliquot of 100 μL was then placed into each well of a 96-well plate. Etoposide (Calbiochem) and H10, prepared using a published strategy (*11*), were used as positive controls, and the vehicle DMSO was used as a negative control. In the presence and absence of compounds, L1210 cells were incubated for 3 d, and the K-562 cells were incubated for 4 d. The HCT-116 cells were incubated overnight, at which point the compounds were added and incubated for 3 d. Next, 10 μL of 5 mg mL^{-1} 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Molecular Probes, now Invitrogen) in media was added to each well and incubated for 2 h (L1210 and K-562) or 3 h (HCT-116). The GI_{50} values were then interpreted on the basis of the bioreduction of MTT into a formazan product. A Molecular Devices Spectramax 190 microplate reader was used to measure the absorbance at 570 and 650 nm to quantify the concentration of formazan present (proportional to the number of living cells). GI_{50}'s were calculated using the HN-NonLin program. Serial dilutions of the compounds were performed in triplicate, and in general the observed errors were $\pm$5 μM for GI_{50} values $>$50 μM, $\pm$0.5 μM for values of 1–50 μM, and $\pm$0.1 μM for values $<$1 μM. The GI_{50}'s reported in Table 1 are the average of the three replicates for each compound.

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Supporting Information Available: This material is available free of charge *via* the Internet.

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