Journal of Medicinal Chemistry

Article

pubs.acs.org/jmc

Synthesis and Biological Evaluation of 1,4-Diaryl-2-azetidinones as Specific Anticancer Agents: Activation of Adenosine Monophosphate Activated Protein Kinase and Induction of Apoptosis†

Farida Tripodi,[‡] Roberto Pagliarin,*[,§] Gabriele Fumagalli,[§] Alessandra Bigi,[‡,⊥] Paola Fusi,[‡] Fulvia Orsini,[§] Milo Frattini,[∥] and Paola Coccetti*[,‡]

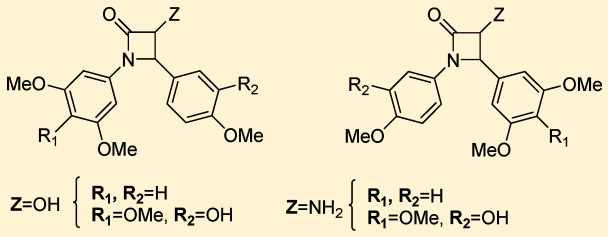
[‡]Department of Biotechnology and Biosciences, University of Milan-Bicocca, Piazza della Scienza 2, 20126 Milan, Italy

[§]Department of Organic and Industrial Chemistry, University of Milan, Via Venezian 21, 20133 Milan, Italy

[∥]Institute of Pathology, Via in Selva 24, 6600 Locarno, Switzerland

Supporting Information

ABSTRACT: A series of novel 1,4-diaryl-2-azetidinones were synthesized and evaluated for antiproliferative activity, cell cycle effects, and apoptosis induction. Strong cytotoxicity was observed with the best compounds (±)-*trans*-**20**, (±)-*trans*-**21**, and enantiomers (+)-*trans*-**20** and (+)-*trans*-**21**, which exhibited IC_{50} values of 3−13 nM against duodenal adenocarcinoma cells. They induced inhibition of tubulin polymerization and subsequent G2/M arrest. This effect was accompanied by activation of AMP-activated protein kinase (AMPK), activation of caspase-3, and induction of apoptosis. Additionally, the most potent compounds displayed antiproliferative activity against different colon cancer cell lines, opening the route to a new class of potential therapeutic agents against colon cancer.



INTRODUCTION

Natural compounds are often used in traditional medicine for their different and multiple therapeutic effects.[1] Among them, stilbene-based compounds are widely distributed in nature and show a wide range of biological activities. Combretastatins (Figure 1), a group of polyhydroxylated stilbenes isolated from the South African tree *Combretum caffrum*, were shown to inhibit the formation of the mitotic spindle by binding to the colchicine-binding site of tubulin.[2] In addition they have also shown antivascular properties in vivo.[3] In particular combretastatin A-4 (CA-4) exhibits potent anticancer activity against a panel of human cancer cells including multidrug resistant ones.[4] Two problems, however, have limited their use as therapeutic agents for a long time: their low water-solubility and cis/trans isomerization which may occur during storage and administration, causing a dramatic loss of activity. The first problem has been overcome by a water-soluble phosphate prodrug (CA-4P, zybrestat, fosbretabulin) that is currently under investigation in human clinical trials (phase III) as an anticancer drug.[5,6] The second problem has been approached by designing a variety of conformationally restricted cis-locked analogues. Cis-restricted combretastatins may be obtained by introducing a five-membered ring (carbocylic or heterocyclic) in place of the olefinic bond. Besides the prevention of cis/trans isomerization, these analogues may have the additional advantage that a proper heterocyclic system might improve the therapeutic potential of these drugs. A large number of modified rings (combretazolones, furanones, cyclopentenones, isoxazolines, isoxazoles, thiazoles, triazoles, tetrazoles, pyrazoles) have been reported as replacements for the cis-double bond and have been reviewed by different authors.[4,7,8] Among them, β-lactam derivatives, which are scaffolds with a similar spatial arrangement between the two phenyl rings as observed in the CA-4 molecule, have also been found to have less toxicity but increased anticancer activity.[9] In addition, a few groups studied the mechanism of action of anticancer polyaromatic β-lactams. In particular, Banik and colleagues showed that some β-lactam derivatives exhibited cytotoxicity against the colon cancer cell line HT-29 with an increase of G2 phase cells 24 h after the treatment.[10] Parallel to this group, other researchers showed that some β-lactam derivatives induced DNA damage, inhibited DNA replication, and activated the apoptotic death program in human leukemic Jurkat T cells.[11] Importantly, one of these derivatives inhibited proliferation and induced apoptosis in various human solid tumor cell lines (breast, prostate, and head-and-neck).[11] Several authors have recently proposed products that maintain the structural elements of the combretastatin, such as the presence of 3,4,5-trimethoxyphenyl moiety. Sun et al.[12] have prepared compounds **3**−**5** (Figure 1) in the cis geometry only; compound **4** exhibits an IC_{50} of 29.19 nM against human renal adenocarcinoma. O'Boyle et al. have proposed trans derivatives **6**[13] and **9**−**13**[9] in which in position 3 is noted the presence of a heterocyclic residue (**6**) or a phenyl group (**9**) or a *p*-fluoro (**10**), a *p*-hydroxyl, or a *p*-aminophenyl group (**11**−**13**). In particular, compound **12** shows an IC_{50} of

Received: October 7, 2011
Published: February 13, 2012

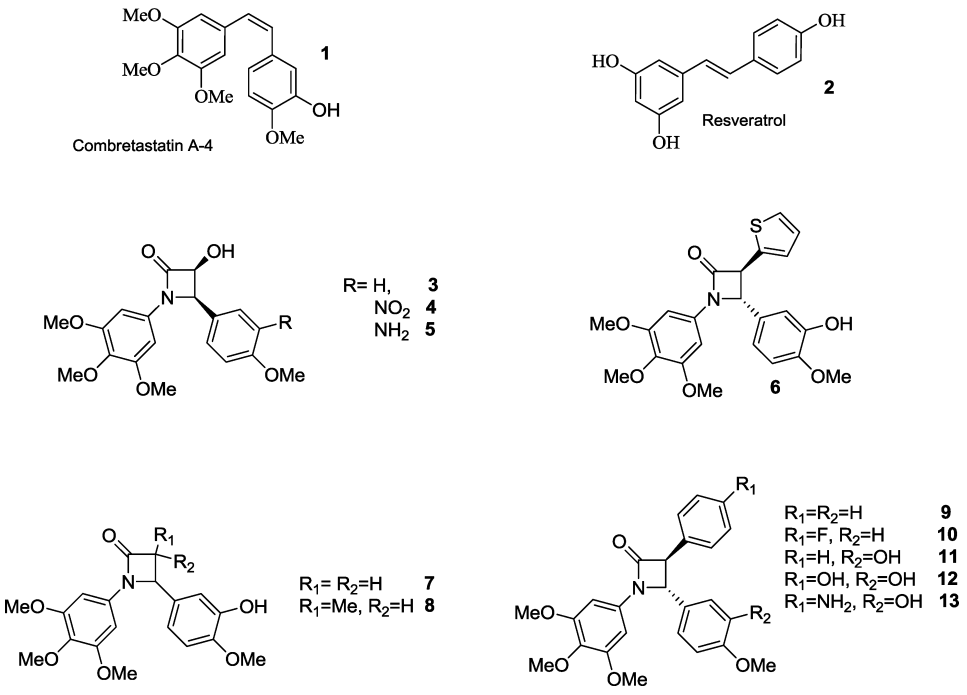


Figure 1. Combretastatin A-4, resveratrol, and anticancer β-lactams.

0.8 nM on human MCF-7 breast cancer cells. Carr et al.[14] synthesized β-lactams without substituents (**7**) or with the presence of one (**8**) or two methyl groups in position 3 of the four-membered ring. Compound **8**, as a mixture of cis/trans stereoisomers tested on MCF-7 cells, has an IC_{50} of 39 nM.

On this base, we focused our work on the preparation of a small library of 1,4-diaryl-2-azetidinones bearing a hydroxyl or an amino group in position 3 of the four-membered ring.[15] We justify the presence of these two substituents in position 3 with the requirement to improve water solubility of the final products.[16] In addition, these groups could be further derivatized, without affecting the aromatic rings, with molecules that enhance water solubility (phosphates) or with specific ligands useful to selectively target tumor cells. We designed four series of compounds that mimic the natural compound CA-4. The first series has a 3,4,5-trimethoxyphenyl residue in position 1 coupled with a 4-methoxy-3-hydroxyphenyl moiety in position 4 of the azetidinone ring and a hydroxyl group (**21**) or an amino group in position 3 (**29**) (Scheme 1). In the second series a 3,5-dimethoxyphenyl substituent is coupled with a 4-methoxyphenyl group in position 4 of the four-membered ring (**20**, **28**, Scheme 1), a substitution pattern also present in resveratrol (Figure 1), the 3,4′,5-trihydroxystilbene identified as a cancer chemopreventive agent.[17,18] The other two series have the inverted aryl moieties of the first (**25**, **33**, Scheme 1) and second series (**24**, **32**, Scheme 1), respectively.

The natural compound CA-4 acts as an activator of either adenosine monophosphate activated protein kinase (AMPK)[19] or of the yeast homologous Snf1/AMPK.[20] In the activated state, AMPK down-regulates several anabolic enzymes and thus shuts down the ATP-consuming metabolic pathways.[21] AMPK is activated upon phosphorylation on Thr172 by its upstream kinase STK11 (LKB1) in response to an increase in cellular AMP/ATP ratio.[22] AMPK activation is cytotoxic to various cancer cell types and inhibits tumor growth,[23,24] supporting AMPK as a tumor suppressor and a potential target for cancer therapy and chemoprevention.[25] Interestingly, many chemotherapic drugs can activate AMPK, mediating apoptotic effects; for example, a recent paper suggests that AMPK activation is a key player for doxorubicin-induced cancer cell apoptosis.[26]

In this study we have found that the new 1,4-diaryl-2-azetidinones show specific antiproliferative activity against duodenal and colon cancer cells, through activation of AMPK and induction of the apoptotic pathway.

CHEMISTRY

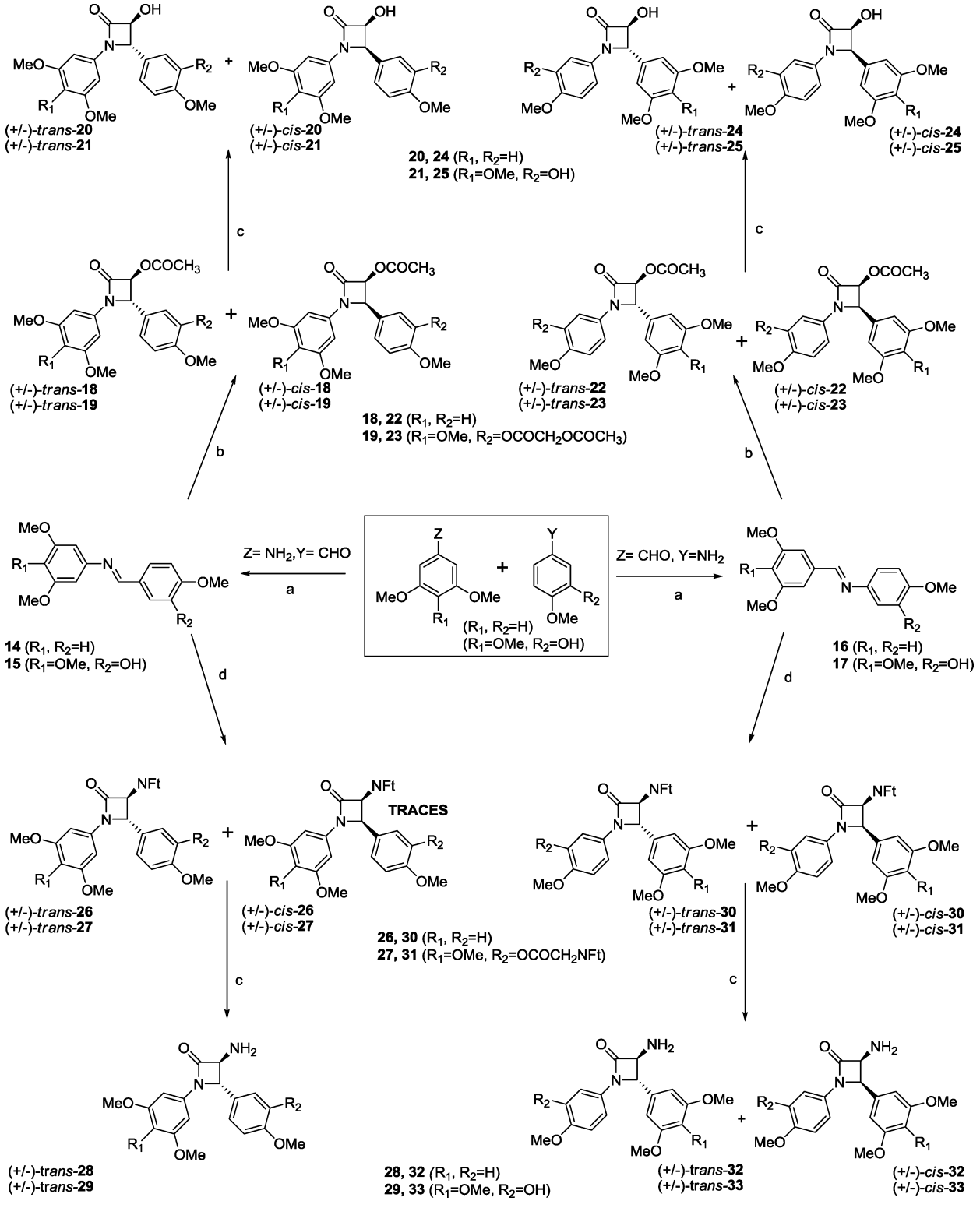
The synthetic route to β-lactam analogues of combretastatins and resveratrol, illustrated in Scheme 1, is based on the Staudinger approach, which is a [2 + 2]-cycloaddition reaction, between properly substituted imines and ketenes, the latter easily obtained "in situ" from the corresponding acid chlorides under basic conditions. The preparation of the imine precursors **14**−**17** was achieved in very good yields (94−98%) by condensation of the properly substituted aldehydes and anilines in ethanol, following two different workup procedures depending on the imine solubility (Scheme 1).

The ketene precursor acetoxyacetyl chloride was used for the preparation of compounds **20**, **21**, **24**, **25**, while phthalylglycyl chloride was used for the preparation of compounds **28**, **29**, **32**, **33**. Among all the synthesized compounds, only compounds **26** and **27** have been obtained in traces, and after purification, the poor yields did not allow the execution of activity tests.

In order to modulate the stereochemical outcome of the reaction, two experimental protocols have been optimized to prepare azetidinones **18**−**33** (Scheme 1). It is indeed well-known that besides the type and number of substituents on both the imine and the ketene, the stereochemistry of the products of the Staudinger cycloaddition is significantly affected by the experimental reaction conditions such as solvent choice, temperature, and order of addition of the reagents.[27] A mixture of racemic cis and trans cycloadducts was obtained by dropwise addition of a 5-fold excess of acid chloride to a methylene chloride solution of imine and TEA. Stereochemistry was

dx.doi.org/10.1021/jm201344a | J. Med. Chem. 2012, 55, 2112−2124

Scheme 1. Synthesis of 3-Hydroxy- and 3-Amino-1,4-diaryl-2-azetidinones[a]



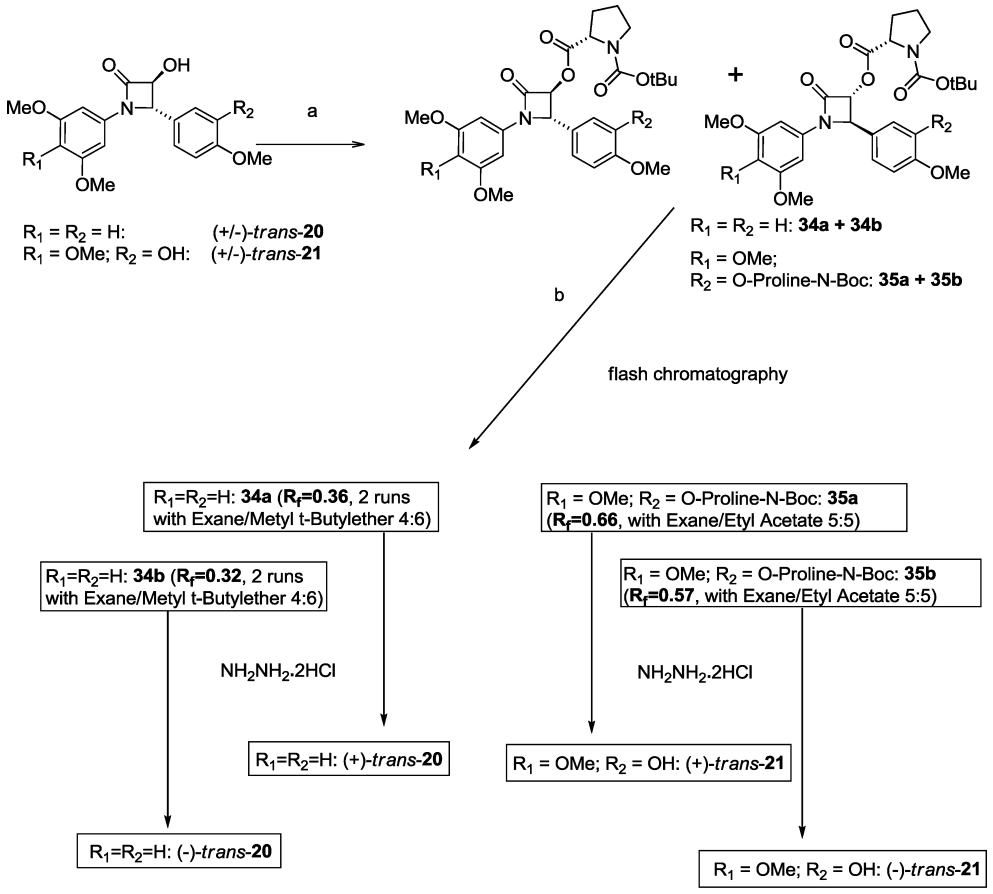
[a]Reagents and conditions: (a) Na_2SO_4, ethanol, 25 °C, 48 h; (b) CH_3COOCH_2COCl, Et_3N, anhydrous CH_2Cl_2, 25 °C, 24 h; (c) $NH_2NH_2{\cdot}2HCl$, Et_3N, MeOH, reflux, 4 h; (d) $FtNCH_2COCl$, Et_3N, anhydrous CH_2Cl_2, 25 °C, 24 h.

assigned by measuring the ^3J coupling constants between H-3 and H-4 (cis = 4−5 Hz, trans = 1−2 Hz).

With the aim of obtaining mainly the more active trans stereoisomers, a different protocol was used, changing solvent, temperature, and order of addition of the reagents.[28] In particular the imine and the ketene precursor, 2-acetoxy acetyl chloride, were mixed at ice temperature, then heated at 100 °C before adding TEA to afford, as racemic mixture, the trans cycloadduct only, albeit in lower yields. The diastereoisomeric cis−trans mixture was separated by flash chromatography.

dx.doi.org/10.1021/jm201344a | J. Med. Chem. 2012, 55, 2112−2124

Scheme 2. Resolution of (±)-3,4-*trans*-3-Hydroxy-1,4-diaryl-2-azetidinones[a]



[a]Reagents and conditions: (a) L-*N*-Boc- proline, DIPEA, HBTU, anhydrous acetonitrile, 24 h; (b) $NH_2NH_2 \cdot 2HCl$, Et_3N, MeOH, reflux, 4 h.

The resolution of the racemic trans mixture of **20** and **21** was tested using different strategies. The best results were obtained using, as chiral solving reagent, N-protected proline as illustrated in Scheme 2. The resulting diastereoisomeric mixtures were then separated by flash chromatography using a properly selected elution system. After the cleavage of the proline moiety, the enantiomeric purity was verified to be 98% via chiral HPLC analysis by comparison with the corresponding racemic mixtures.

RESULTS

Compounds (±)-*trans*-20, (±)-*trans*-21, (+)-*trans*-20, and (+)-*trans*-21 Show in Vitro Antiproliferative Activities against Cancer Cell Lines. The new β-lactam compounds were tested in HeLa cells (cervical adenocarcinoma) in order to evaluate their efficacy as antiproliferative agents. The cells were treated with 10 μM of each compound, and their viability was measured after 48 h by the MTT assay. The analyses revealed that (±)-*cis*-**20**, (±)-*trans*-**20**, (±)-*cis*-**21**, (±)-*trans*-**21**, (±)-*cis*-**25**, and (±)-*trans*-**25** were the most promising, showing an effect on cell viability similar to that observed with combretastatin A4 (Figure S1 of Supporting Information). We thus decided to evaluate the antiproliferative effect of these compounds on small bowel adenocarcinoma (SBA, HuTu-80 cancer cells), using CA-4 and compounds **4** and **5**, previously described in literature,[12] as controls. All compounds exhibited IC_{50} values ranging from a few nanomolar to 2.5 μM (Table 1). Compounds (±)-*trans*-**20** and (±)-*trans*-**21** showed the most potent antitumor effect toward HuTu-80 cells, with IC_{50} of about 9 and 13 nM, respectively (Table 1). Enantiomerically pure (+)-*trans*-**20**,

Table 1. IC_{50} of (±)-*cis*-20, (±)-*trans*-20, (+)-*trans*-20, (−)-*trans*-20, (±)-*cis*-21, (±)-*trans*-21, (+)-*trans*-21, (−)-*trans*-21, (±)-*cis*-25, (±)-*trans*-25, 4, 5, and CA-4 in HuTu-80 Cells Measured by MTT Assay after 72 h of Treatment[a]

compd	IC_{50}, nM (HuTu-80 cells, 72 h)
(±)-*cis*-**20**	124.6 ± 27.01
(±)-*trans*-**20**	9.05 ± 1.57
(+)-*trans*-**20**	8.02 ± 0.71
(−)-*trans*-**20**	83.55 ± 14.33
(±)-*cis*-**21**	28.79 ± 3.36
(±)-*trans*-**21**	13.36 ± 1.47
(+)-*trans*-**21**	3.05 ± 1.42
(−)-*trans*-**21**	589.9 ± 98.71
(±)-*cis*-**25**	2549.00 ± 383.25
(±)-*trans*-**25**	405.5 ± 48.65
4	528.55 ± 27.93
5	101.17 ± 6.75
CA-4	1.37 ± 0.13

[a]IC_{50} values are the compound concentrations required to inhibit cell proliferation by 50%. Data are expressed as the mean ± standard deviation from the dose−response curves of at least three independent experiments performed in triplicate.

dx.doi.org/10.1021/jm201344a | J. Med. Chem. 2012, 55, 2112−2124

Table 2. IC_{50} of (±)-*trans*-**20**, (+)-*trans*-**20**, (±)-*trans*-**21**, (+)-*trans*-**21**, and CA-4 in SW48, SW480, SW620, T84, MCF-7, and SKNBE Cell Lines Measured by MTT Assay after 72 h of Treatment[a]

	IC_{50}, nM					
compd	SW48	SW480	SW620	T84	MCF-7	SKNBE
(±)-*trans*-**20**	12.05 ± 0.92	24.17 ± 5.84	6.6 ± 0.49	5.73 ± 0.47	5.19 ± 1.65	2.25 ± 0.36
(+)-*trans*-**20**	11.06 ± 1.61	16.74 ± 4.01	8.58 ± 0.18	6.77 ± 2.28	4.01 ± 2.43	1.12 ± 0.22
(±)-*trans*-**21**	15.19 ± 3.28	22.14 ± 4.55	13.63 ± 4.34	45.37 ± 4.70	6.14 ± 2.73	4.20 ± 1.82
(+)-*trans*-**21**	9.81 ± 3.02	12.53 ± 3.58	3.37 ± 1.80	20.30 ± 2.55	4.47 ± 0.96	1.32 ± 0.50
CA-4	3.32 ± 1.61	3.63 ± 1.65	1.52 ± 0.13	23.43 ± 5.81	1.11 ± 0.13	0.58 ± 0.14

[a]IC_{50} values are the compound concentrations required to inhibit cell proliferation by 50%. Data are expressed as the mean ± standard deviation from the dose−response curves of at least three independent experiments performed in triplicate.

(−)-*trans*-**20**, (+)-*trans*-**21**, and (−)-*trans*-**21** were obtained and tested on the same cells. It is noteworthy that enantiomer (+)-*trans*-**20** retained the same cytotoxic activity as the racemic solution ((±)-*trans*-**20**). In contrast, the IC_{50} value of (−)-*trans*-**20** was about 10 times higher than that of (+)-*trans*-**20** (Table 1). Remarkably, the enantiomer (+)-*trans*-**21** showed an IC_{50} of 3 nM, whereas enantiomer (−)-*trans*-**21** was less active with an IC_{50} close to 600 nM (Table 1).

Compounds (±)-*trans*-**20**, (±)-*trans*-**21**, (+)-*trans*-**20**, and (+)-*trans*-**21** were additionally tested against a panel of six human cancer cell lines, including colon cancer cells (SW48, SW480, SW620, T84), the breast cancer cell line MCF-7, and the neuroblastoma cell line SKNBE (Table 2). The compounds retained nanomolar cytotoxic activity against all cancer cells, and enantiomerically pure compounds (+)-*trans*-**20** and (+)-*trans*-**21** were more active than their respective racemic mixtures in most cell lines (Table 2). In contrast, normal FHs74 intestinal cells were not affected and only a marginal inhibitory effect was observed on normal CCD841CoN colon cells at 10 μM (data not shown), suggesting a cancer-specific activity of these compounds.

Compounds (±)-*trans*-20, (±)-*trans*-21, (+)-*trans*-20, and (+)-*trans*-21 Induce Apoptotic Cell Death. The effects of (±)-*trans*-**20** and (±)-*trans*-**21** on cell cycle progression were examined by analyzing the DNA content of HuTu-80 cells treated with 30 nM of these compounds for 48 and 72 h (0.1% DMSO and CA-4 were used as negative and positive controls, respectively). Both (±)-*trans*-**20** and (±)-*trans*-**21** caused a large increase of cells in the G2/M phase which is accompanied by a corresponding reduction in the G1 phase (Figure S2A of Supporting Information), as reported for the control CA-4.[29,30]

These results suggest that the compounds may act by targeting the microtubules, like CA-4.[1,2] The effects of the more active compounds (±)-*trans*-**20**, (±)-*trans*-**21**, (+)-*trans*-**20**, and (+)-*trans*-**21** on the assembly of purified porcine tubulin were then evaluated. The ability of CA-4 to effectively inhibit tubulin polymerization was assessed as a positive control (Table 3). In the assembly assay, all the azetidinone compounds showed an IC_{50} value ranging from 1.3 to 3.8 μM, indicating that one of the molecular targets of the active azetidinones is tubulin. Strikingly, the enantiomerically pure compounds (+)-*trans*-**20** and (+)-*trans*-**21** were more potent than their racemic mixtures, with an IC_{50} values close to that of CA-4 (Table 3 and as previously reported[8]).

In addition, treatment of cells with (±)-*trans*-**20** and (±)-*trans*-**21** was able to induce the time-dependent appearance of a hypodiploid peak (sub-G1) indicative of cell death (Figure S2A of Supporting Information). Since sub-G1 cells do not necessarily indicate apoptosis, we investigated the activation

Table 3. Inhibition of Tubulin Polymerization in Response to Azetidinone Compounds[a]

compd	tubulin polymerization IC_{50} (μM)
(±)-*trans*-**20**	3.60 ± 0.51
(+)-*trans*-**20**	1.30 ± 0.20
(±)-*trans*-**21**	3.80 ± 0.60
(+)-*trans*-**21**	1.61 ± 0.42
CA-4	1.32 ± 0.15

[a]IC_{50} values are the compound concentrations required to inhibit tubulin polymerization by 50%. DMSO (0.2% v/v) was used as a vehicle control. Data are expressed as the mean ± standard deviation from the dose−response curves of at least two independent experiments performed in triplicate.

of the caspase pathway. HuTu-80 cells were treated with 30 nM (±)-*trans*-**20** and (±)-*trans*-**21** for 24 h, and caspase-3 and PARP (poly ADP-ribose polymerase) cleavage was examined by Western blot analysis (Figure 2 and S2B of Supporting

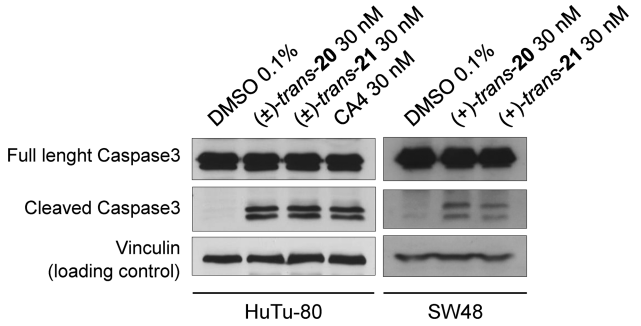


Figure 2. The new β-lactams induce apoptosis in cancer cells. HuTu-80 cells were treated with 30 nM (±)-*trans*-**20** and (±)-*trans*-**21** for 24 h, and SW48 cells were treated with 30 nM (+)-*trans*-**20** and (+)-*trans*-**21** for 16 h. Total protein extracts were subjected to Western blot analysis to detect caspase-3. Vinculin was used as loading control. Treatments with 0.1% DMSO and with 30 nM CA-4 were used as negative and positive controls, respectively.

Information). Treatment with (±)-*trans*-**20** and (±)-*trans*-**21** strongly led to extensive formation of the typical fragments of caspase-3 and PARP, indicative of apoptosis induction, similar to that observed with CA-4 (Figure 2 and S2B of Supporting Information).

Since SBA is a model of cancer superimposable on colon cancer cells (CRC),[31−33] we examined the induction of the apoptotic pathway on the SW48 colon cancer cell line, which displayed a strong growth inhibition induced by (+)-*trans*-**20** and (+)-*trans*-**21** (Table 2). On the basis of the evidence of the similar molecular profiles of CRC and SBA,[31−33] we expected

dx.doi.org/10.1021/jm201344a | J. Med. Chem. 2012, 55, 2112−2124

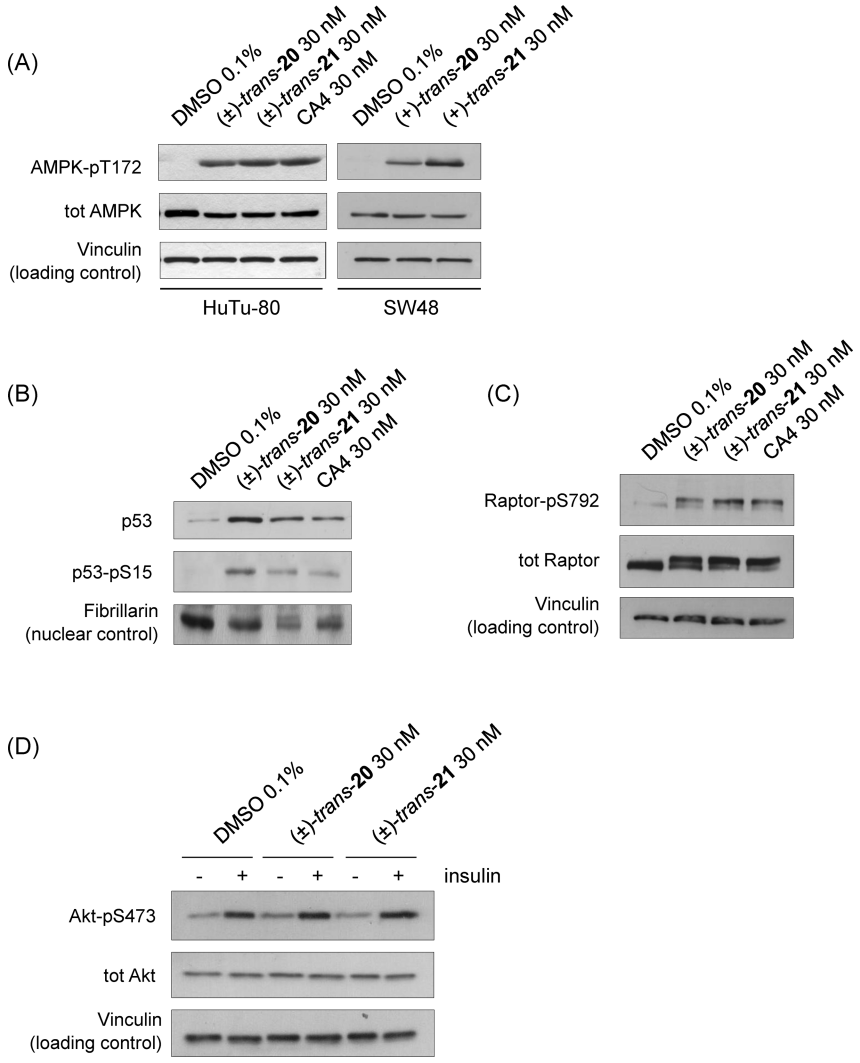


Figure 3. Compounds (±)-*trans*-**20**, (±)-*trans*-**21**, (+)-*trans*-**20**, and (+)-*trans*-**21** induce AMPK activation. (A) HuTu-80 cells were treated with 30 nM (±)-*trans*-**20** and (±)-*trans*-**21** for 24 h, and SW48 cells were treated with 30 nM (+)-*trans*-**20** and (+)-*trans*-**21** for 16 h. Total protein extracts were subjected to Western blot analysis to assay pT172-AMPK and total AMPK levels. (B) HuTu-80 cells were treated with 30 nM (±)-*trans*-**20** and (±)-*trans*-**21** for 24 h, and nuclear protein extracts were subjected to Western blot analysis to assay pS15-p53 and total p53 levels. Fibrillarin was used as nuclear control. (C) HuTu-80 cells were treated with 30 nM (±)-*trans*-**20** and (±)-*trans*-**21** for 24 h, and total protein extracts were subjected to Western blot analysis to assay pS792-Raptor and total Raptor levels. (D) HuTu-80 cells were treated with (±)-*trans*-**20** and (±)-*trans*-**21** (30 nM) for 24 h and then mock treated or treated with 10 μg/mL insulin for 10 min. Total protein extracts were subjected to Western blot analysis to assay pS473-Akt and total Akt levels. Vinculin was used as loading control. Treatments with 0.1% DMSO and with 30 nM CA-4 were used as negative and positive controls, respectively.

to find similar results. Indeed, treatment with (+)-*trans*-**20** and (+)-*trans*-**21** induced activation of caspase-3 (Figure 2). These data clearly indicate that (±)-*trans*-**20**, (±)-*trans*-**21**, (+)-*trans*-**20**, and (+)-*trans*-**21** induce apoptotic cell death in cancer cells.

Compounds (±)-*trans*-20, (±)-*trans*-21, (+)-*trans*-20, and (+)-*trans*-21 Induce AMPK Activation Pathway. Previous studies showed that CA-4 activates AMPK in HepG2 cells.[19] We thus examined whether AMPK was also activated by the new azetidinone derivatives, which are structural analogues of CA-4.[12] Compounds (±)-*trans*-**20** and (±)-*trans*-**21** induced phosphorylation of AMPK on Thr172 in HuTu-80 cells, as well as (+)-*trans*-**20** and (+)-*trans*-**21** in SW48 cells (Figure 3A). Since AMPK acts on the tumor-suppressor p53 leading to apoptotic cell death,[34,35] our analysis was further deepened, investigating the level and the phosphorylation state of the tumor suppressor protein p53. It is known that AMPK activation induces phosphorylation of p53 on Ser15, which is required for AMPK-dependent cell cycle arrest.[34,36] Compounds (±)-*trans*-**20** and (±)-*trans*-**21**, as well as the control CA-4, induced phosphorylation of p53 on Ser15. Moreover, p53 was found to be accumulated (Figure 3B). Another downstream effector of AMPK is the mTOR binding partner Raptor, whose phosphorylation by AMPK is required for the inhibition of mTORC1 and cell cycle arrest induced by energy stress.[37] Raptor was found to be phosphorylated on Ser792 upon treatment with (±)-*trans*-**20** and (±)-*trans*-**21** (Figure 3C).

It was shown that other AMPK activators, such as metformin and AICAR, induce diminished expression of pSer473-Akt,[38] which plays a key role in multiple cellular processes such as cell growth, cell survival, and cell cycle progression.[39] We thus tested the effect of (±)-*trans*-**20** and (±)-*trans*-**21** on Akt phosphorylation, both in unstimulated cells and upon insulin stimulation. No differences in the level of pSer473-Akt were

dx.doi.org/10.1021/jm201344a | J. Med. Chem. 2012, 55, 2112−2124

observed, neither in the basal condition nor upon insulin stimulation, suggesting that the inhibitory effect of the new azetidinone compounds is not mediated by the Akt upstream activation (Figure 3D).

DISCUSSION AND CONCLUSIONS

In the present article we report the synthesis of new azetidinone derivatives, with a strong antiproliferative activity against cancer cells (Table 1 and 2). In our activity test, trans azetidinone derivatives show better results than cis isomers, and they can be prepared in good yields by thermodynamic reaction conditions.[27] Moreover, the presence of a hydroxyl group at position 3 of the azetidinone ring gives a water solubility 5 times more elevated for (±)-*trans*-**21** with respect to the natural product combretastatin A4.[16,40] The molecules (±)-*trans*-**20** and (±)-*trans*-**21** were identified as the most potent compounds of the series having nanomolar activity against several colon cancer cell lines (SW48, SW480, SW620, T84) as well as on the breast cancer cell line MCF-7 and on the neuroblastoma cell line SKNBE. These compounds, resolved in the pure enantiomers, are more active than their respective racemic mixtures in most cell lines, showing a positive correlation between cytotoxic activity and inhibition of tubulin polymerization (Tables 1−3).

Pure enantiomer (+)-*trans*-**21** exhibited an activity 200 times higher than the (−)-*trans*-antipode (Table 1). In keeping with literature data,[12] our results confirm that compounds with the 3,4,5-trimethoxy or 3,5-dimethoxyphenyl ring on the N-1 position of azetidinone ring are significantly more active than compounds with polymethoxylated moieties on the C-4 position.

Our data show that the mechanism of the antitumoral action exerted by these new azetidinone compounds relies on the inhibition of tubulin polymerization and the stimulation of the apoptotic pathway, with the cleavage of the effector caspase-3 and the PARP protein (Figure 2 and Figure S2 of Supporting Information). It is of high relevance that our compounds induced AMPK activation (Figure 3), since AMPK is a novel therapeutic target for cancer and metabolic diseases. Indeed, when physiologically or pharmacologically activated, AMPK acts in a tumor suppressor-like fashion.[41] In addition, AMPK inhibits the mTOR pathway through direct phosphorylation of the mTOR-associated factor Raptor and induces cell cycle arrest and/or apoptosis through phosphorylation of p53.[22] The mTOR signaling pathway is known to play important roles in the protein synthesis machinery.[42] Therefore, when AMPK is activated, cell growth and proliferation might be inhibited as a result of limitations in protein synthesis.

The ability of these compounds to inhibit cancer cell proliferation can be of particular significance against small bowel adenocarcinoma (SBA) and colorectal cancer diseases (CRC), which display similar risk factors and share the same carcinogenetic process,[31−33] since compounds (±)-*trans*-**20** and (±)-*trans*-**21**, as well as their enantiomers (+)-*trans*-**20** and (+)-*trans*-**21**, showed a strong antiproliferative activity against these cancer cells (Tables 1 and 2). In addition, they showed only a slight inhibitory effect on the two normal cell lines analyzed (FHs74 intestinal cells and CCD841CoN colon cells), even at very high concentrations (about 1000-fold higher than the IC_{50} concentration estimated on most cancer cell lines tested, data not shown). This specific anticancer activity is of great interest considering that chemotherapies are limited by their frequent and potentially severe dose-limiting toxicities. Thus, we suggest that the new 1,4-diaryl-2-azetidinones are promising novel anticancer drugs to be tested in preclinical cancer models of CRC.

EXPERIMENTAL SECTION

Chemistry: Experimental Methods. All commercially available reagents were purchased by Sigma-Aldrich and used without further purification. Preparative separations were usually performed by flash column chromatography on silica gel (Merck grade 9385). Thin-layer chromatography was conducted using silica gel (Merck, 10 cm × 5 cm, silica gel 60 F254). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC 200 spectrometer at 200 and 50.3 MHz, respectively, in $CDCl_3$ solution unless stated otherwise, and chemical shifts were represented as δ values relative to the internal standard TMS. ESI mass spectra were recorded on Bruker Esquire 3000 Plus. The synthesized compounds submitted to biological tests have a purity of ≥95% determined by HPLC (Phenomenex, Nucleosil 250 mm × 3.2 mm column, 5 μm, C18; mobile phase 0.05 M phosphate buffer, pH 7/acetonitrile, 7:3; flow rate 1.5 mL/min), and they have been filtered in sterile atmosphere with 0.20 μm filters. Chiral liquid chromatography was carried out on selected compounds using a DAICEL Chirapak IB 4.0 mm column and Hewlett-Packard 1050 HPLC instrument. The organic mobile phase was 2-propanol/Exane, 20:80. The flow rate was 0.8 mL/min, and detection was carried out at 225 nm. Water solubility of (±)-*trans*-**20** and (±)-*trans*-**21** have been obtained after stirring a suspension of each compound for 2 h at 30 °C. They have been further centrifuged, and the liquid phase was analyzed by HPLC (Supelcosil LC18, 250 mm × 4.6 mm, 5 μm; mobile phase 0.05 M phosphate buffer, pH 7/acetonitrile, 5:5; flow rate 1 mL/min). The concentrations have been calculated by intercepting the peak area values with the calibration curves obtained by standard solutions of compounds **20** and **21** in acetonitrile.

4-Methoxybenzylidene-(3,5-dimethoxyphenyl)amine 14. Dry Na_2SO_4 (4 g) was added to a solution of 3,5-dimethoxyaniline (3.0 g, 19.6 mmol) and 4-methoxybenzaldehyde (2.67 g, 19.6 mmol) in ethanol (6 mL), and the mixture was stirred at room temperature for 48 h. The mixture was filtered and the solvent removed under reduced pressure to afford the imine **14** as a brown oil (5.0 g, 18.4 mmol, 94% yields). ^{1}H NMR ($CDCl_3$): δ = 8.41 (1H, s), 7.86 (2H, d, J = 8.8 Hz), 7.00 (2H, d, J = 8.8 Hz), 6.40 (2H, d, J = 2 Hz), 6.36 (1H, t, J = 2 Hz), 3.88 (3H, s), 3.83 (6H, s). EI-MS (m/z): 271 (M^+).

3-Hydroxy-4-methoxybenzylidene-(3,4,5-trimethoxyphenyl)amine 15. Dry Na_2SO_4 (4 g) was added to a solution of 3,4,5-trimethoxyaniline (1.9 g, 10.1 mmol) and 3-hydroxy-4-methoxybenzaldehyde (1.5 g, 10.1 mmol) in ethanol (6 mL), and the mixture was stirred at room temperature for 48 h to afford a yellow solid. The imine **15** was a solid mixed with Na_2SO_4. The mixture was filtered, and the solid was washed with cold ethanol (10 mL). The solid was suspended in methylene chloride and filtered to separate the solubilized imine **2** from Na_2SO_4 (3.1 g, 9.9 mmol, 98% yield). ^{1}H NMR ($CDCl_3$): δ 8.41 (1H, s), 7.63 (1H, s), 7.46 (1H, d, J = 7.2 Hz), 6.96 (1H, d, J = 7.2 Hz), 6.53 (2H, s), 5.77 (1H, s), 3.99 (3H, s), 3.92 (6H, s), 3.88 (3H, s). EI-MS (m/z): 318 (M^+).

3,5-Dimethoxybenzylidene-4-methoxyphenylamine 16. This compound has been prepared following the same methodology described for **14** (92% yield). δ = 8.41 (1H, s), 7.26 (2H, d, J = 8.9 Hz), 7.07 (2H, d, J = 2.0 Hz), 6.95 (2H, J = 8.9 Hz), 6.59 (1H, t, J = 2.0 Hz), 3.88 (6H, s), 3.85 (3H, s). EI-MS (m/z): 271 (M^+).

3,4,5-Trimethoxybenzylidene-(3-hydroxy-4-methoxyphenyl)amine 17. This compound has been prepared following the same methodology described for **15** (80% yield). δ = 8.36 (1H, s), 7.16 (2H, s), 6.92 (1H, d, J = 2.3 Hz), 6.89 (1H, d, J = 8.5 Hz), 6.81 (1H, dd, J = 2.3, 8.5 Hz), 5.86 (1H, s), 3.95 (3H, s), 3.92 (9H, s). EI-MS (m/z): 318 (M^+).

General Methods for the Synthesis of Azetidin-2-ones (Trans + Cis). Procedure a. (±)-3,4-*trans*-1-(3,5-Dimethoxyphenyl)-3-hydroxy-4-(4-methoxyphenyl)azetidin-2-one ((±)-*trans*-20) and (±)-3,4-*cis*-1-(3,5-Dimethoxyphenyl)-3-hydroxy-4-(4-methoxyphenyl)azetidin-2-one ((±)-*cis*-20). A solution of 2-acetoxyacetyl chloride (7.44 g, 54.5 mmol) in anhydrous

methylene chloride (30 mL) was added dropwise at 0 °C under nitrogen to a stirred solution of imine **14** (2.97 g, 10.9 mmol) and TEA (17.6 g, 24.3 mL, 174 mmol) in anhydrous methylene chloride (90 mL). The solution was allowed to reach room temperature and then stirred for 24 h. The solvent was concentrated under reduced pressure and the residue was purified by flash chromatography (silica gel; eluent *n*-hexane/ethyl acetate, 1/1) to afford a mixture of the two diastereoisomers (±)-*trans*-**18** and (±)-*cis*-**18** as a white amorphous solid (1.67 g, 4.5 mmol, 41% yield). A sample of this mixture was further purified by gradient flash chromatography with *n*-hexane/ethyl acetate, and the two diastereoisomers (±)-*trans*-**18** and (±)-*cis*-**18** were identified by analytical techniques.

(±)-*trans*-**18**. ^{1}H NMR ($CDCl_3$) δ: 7.23 (2H, d, J = 7.7 Hz), 6.85 (2H, d, J = 7.7 Hz), 6.42 (2H, d, J = 2.3 Hz), 6.20 (1H, t, J =2.3 Hz), 5.30 (1H, d, J = 2 Hz), 4.82 (1H, d, J=2 Hz), 3.80 (3H, s), 3.70 (6H, s), 2.17 (3H, s). ^{13}C NMR ($CDCl_3$) δ: 169.81, 162.20, 161.30, 160.30, 138.66, 127.80, 127.22, 114.75, 97.10, 96.43, 82.72, 63.74, 55.49, 55.45, 20.59. EI-MS (m/z): 371 (M^+).

(±)-*cis*-**18**. ^{1}H NMR ($CDCl_3$) δ: 7.23 (2H, d, J = 8.7 Hz), 6.87 (2H, d, J = 8.7 Hz), 6.53 (2H, d, J = 2.2 Hz), 6.22 (1H, t, J = 2.2 Hz), 5.89 (1H, d, J = 4.9 Hz), 5.29 (1H, d, J = 4.9 Hz), 3.81 (3H, s), 3.73 (6H, s), 1.74 (3H, s). ^{13}C NMR ($CDCl_3$) δ: 169.81, 162.43, 161.36, 160.15, 138.82, 129.32, 124.05, 114.14, 97.12, 96.31, 77.82, 61. 53, 55.52, 55.49, 20.02. EI-MS (m/z): 371(M^+).

Hydrazine dihydrochloride (3.3 g, 31.5 mmol) was added, at 0 °C and under nitrogen to a stirred solution of (±)-*trans*-**18** and (±)-*cis*-**18** (1.67 g, 4.5 mmol) in methanol (65 mL). TEA (9.11 g, 12.5 mL, 90 mmol) was added dropwise. The mixture was allowed to reach room temperature and then refluxed for 4 h. The solvent was removed at reduced pressure, and the residue was treated with a saturated solution of $KHSO_4$ and extracted with ethyl acetate (3 × 30 mL). The combined organic phases were dried (Na_2SO_4), and the solvent was removed at reduced pressure. The crude residue was purified by gradient flash chromatography (silica gel; eluent *n*-hexane/*tert*-butyl methyl ether from 7/3 to 3/7) to afford the diastereoisomers (±)-*trans*-**20** (0.050 g, 0.15 mmol, 3.3% yield) and (±)-*cis*-**20** (0.246 g, 0.75 mmol, 17% yield) as a colorless amorphous solid.

(±)-*trans*-**20**. ^{1}H NMR ($CDCl_3$) δ: 7.25 (2H, d, J = 8.7 Hz), 6.88 (2H, d, J = 8.7 Hz), 6.42 (2H, d, J = 2.2 Hz), 6.14 (1H, t, J = 2.2 Hz), 4.81 (1H, d, J = 1.8 Hz), 4.69 (1H, d, J = 1.8 Hz), 3.80 (3H,s), 3.67 (6H, s). ^{13}C NMR ($CDCl_3$) δ: 167.77, 161.14, 160.09, 138.67, 128.13, 127.60, 114.45, 97.07, 96.52, 83.77, 65.82, 55.49. EI-MS (m/z): 329 (M^+), 272.

(±)-*cis*-**20**. ^{1}H NMR ($CDCl_3$) δ: 7.26 (2H, d, J = 8.6 Hz), 6.92 (2H, d, J = 8.6 Hz), 6.53 (2H, d, J =2.0 Hz), 6.19 (1H, t, J = 2.0 Hz), 5.21 (1H, d, J = 5.6 Hz), 5.14 (1H, d, J = 5.6 Hz), 3.79 (3H,s), 3.71 (6H, s). ^{13}C NMR ($CDCl_3$) δ: 167.67, 161.33, 160.27, 138,80, 128.90, 124.77, 114.80, 96.93, 96.41, 77.10, 65.69, 55.57. EI-MS (m/z) 329 (M^+).

(±)-3,4-*trans*-3-Hydroxy-4-(3-hydroxy-4-methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one ((±)-*trans*-21) and (±)3,4-*cis*-3-Hydroxy-4-(3-hydroxy-4-methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one ((±)-*cis*-21). Compounds (±)-*trans*-**21** and (±)-*cis*-**21** were prepared following the same methodology described for **20**. Compounds *cis*-**19** and *trans*-**19** were obtained with 60% yield.

(±)-*trans*-**19**. ^{1}H NMR ($CDCl_3$) δ: 7.23 (1H, dd, J = 8.5, 2.2 Hz), 7.14 (1H, d, J = 2.2 Hz), 7.00 (1H, d, 8.5 Hz), 6.53 (2H, s), 5.38 (1H, d, J = 1.7 Hz), 4.68 (2H,s), 4.58 (1H, d, J = 1.7 Hz), 3.85 (3H, s), 3.79 (3H, s), 3.74 (6H,s), 2.19 (3H, s), 1.76 (3H, s). ESI-MS (m/z): 517.

(±)-*cis*-**19**. ^{1}H NMR ($CDCl_3$) δ: 7,22 (1H, dd, J = 8.4, 2.0 Hz), 7.05 (1H, d, J = 2.0 Hz), 6.96 (1H, d, J = 8.4 Hz), 6.57 (2H, s), 5.90 (1H, d, J = 4.8 Hz), 5.28 (1H, d, J = 4.8 Hz), 4.89 (2H, s), 3.82 (3H, s), 3.78 (3H, s), 3.73 (6H, s), 2.19 (3H, s), 1.76 (3H, s). ^{13}C NMR ($CDCl_3$) δ: 170.37, 169.57, 166.00, 161.81, 153.81, 151.71, 139.27, 132.98, 127.17, 124.86, 122.54, 113.28, 95.45, 76.48, 61.20, 61.11, 60.48, 56.32, 20.62, 20.06. ESI-MS (m/z): 517 (M^+).

Compounds **21** were purified by gradient flash chromatography (silica gel; eluent *n*-hexane/*tert*-butyl methyl ether from 6/4 to 2/8). The diastereoisomers (±)-*trans*-**21** (7% yield) and (±)-*cis*-**21** (40% yield) were isolated as colorless amorphous compounds.

(±)-*trans*-**21**. ^{1}H NMR ($CDCl_3$) δ: 6.92−6.85 (3H, m), 6.49 (2H, s), 5.84 (1H, s), 4.72 (2H, bs), 3.87 (3H, s), 3.74 (3H, s), 3.62 (6H, s). ^{13}C NMR ($CDCl_3$) δ: 167.68, 154.05, 147.67, 146.94, 135.39, 133.80, 129.81, 118.80, 112.91, 111.68, 96.14, 84.14, 66.35, 61.57, 55.67. ESI-MS (m/z): 398 (M + Na^+).

(±)-*cis*-**21**. ^{1}H NMR ($CDCl_3$): δ 7.10 (1H, d, J = 8.8 Hz), 7.00−6.95 (2H, m), 6.81 (2H, s), 5.35 (1H, d, J = 5.0 Hz), 5.28 (1H, d, J = 5.0 Hz), 4.00 (3H, s), 3.86 (6H, s), 3.84 (3H, s). ^{13}C NMR ($CDCl_3$) δ:167.25, 153.28, 145.02, 143.25, 133.51, 126.47, 119.17, 114.49, 111.14, 95.13, 76.50, 62.69, 59.65, 54.93. ESI-MS (m/z): 398 (M + Na^+).

General Methods for the Synthesis of Azetidin-2-ones (Trans). Procedure b. (±)-3,4-*trans*-1-(3,5-Dimethoxyphenyl)-3-hydroxy-4-(4-methoxyphenyl)azetidin-2-one ((±)-*trans*-20). The imine **14** (5.0 g, 18.4 mmol) and 2-acetoxyacetyl chloride (2.5 g, 1.97 mL, 18.4 mmol) were dissolved in dry toluene (50 mL), under nitrogen and stirring, at 0 °C. The solution was allowed to reach room temperature and then warmed to 100 °C. Dry TEA (2.0 g, 2.75 mL, 20.2 mmol) was added dropwise. The mixture was warmed at 100 °C for 5 h. The solution was concentrated under reduced pressure and the residue was purified by flash chromatography (silica gel; eluent *n*-hexane/ethyl acetate, 1/1) to afford the diastereoisomer (±)-*trans*-**18** (3.4 g, 9.2 mmol, 50% yield) as an amorphous white solid. Hydrazine dichloride (2.85 g, 27.2 mmol) was added to a stirred solution of *trans*-**18** (2.5 g, 6.8 mmol) in methanol (30 mL) at 0 °C and under nitrogen. TEA (5.5 g, 7.6 mL, 54 mmol) was added dropwise. The mixture was allowed to reach room temperature and then refluxed at for 4 h. The solvent was removed at reduced pressure, and the residue was treated with a saturated solution of $KHSO_4$ and extracted with ethyl acetate (3 × 50 mL). The organic phase was dried over Na_2SO_4, and the solvent, after filtration, was removed at reduced pressure. The crude residue was purified by flash chromatography (silica gel; eluent *n*-hexane/ethyl acetate, 1:1). (±)-*trans*-**18** (2.2 g, 6.8 mmol, 37% yield) was obtained as a white amorphous solid.

(±)-3,4-*trans*-3-Hydroxy-4-(3-hydroxy-4-methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one ((±)-*trans*-21). The compound (±)-*trans*-**21** was prepared following the same methodology described for **20**. The compound *trans*-**19** was obtained with 35% yield. Compound **21** was purified by gradient flash chromatography (silica gel; eluent *n*-hexane/*tert*-butyl methyl ether 1:1). (±)-*trans*-**21** (94% yield) was obtained as a white amorphous solid.

Resolution of Racemic (*trans*)-Azetidin-2-ones. Resolution of (±)-*trans*-20. HBTU (5.6 g, 15 mmol) was added, under nitrogen, to a stirred solution of (±)-*trans*-**20** (2.2 g, 6.8 mmol) and L-Boc-prolina (3.0 g, 14 mmol) in dry acetonitrile (30 mL). DIPEA (72.3 mL, 420 mmol) was added dropwise, and the reaction mixture was stirred for 24 h. The solvent was removed under reduced pressure, and the residue was diluted with water and extracted with methylene chloride (3 × 50 mL). The combined organic extracts were washed with a saturated solution of $KHSO_4$, with a saturated solution of $NaHCO_3$, with brine and then dried (Na_2SO_4). The solvent was removed under reduced pressure and the residue purified by gradient flash chromatography (silica gel; eluent *n*-hexane/ethyl acetate from 8/2 to 6/4) to afford a mixture of the two diastereoisomers **34a** + **34b** as colorless solid (3.1 g, 5.9 mmol, 86% yield). The two diastereoisomers were then separated by gradient flash chromatography (silica gel) using *n*-hexane/*tert*-butyl methyl ether as eluent (*n*-hexane/*tert*-butyl methyl ether from 8/2 to 2/8).

34a. Diastereoisomer with R_f = 0.36; on silica gel TLC plate, two runs, eluent *n*-hexane/*tert*-butyl methyl ether, 4:6. $[\alpha]_D$ +1.25 (*c* 30 mg/10 mL). ^{1}H NMR (DMSO-d_6, 80 °C, 500 MHz) δ: 7.38 (2H, d, J = 8.5 Hz), 6.97 (2H, d, J = 8.5 Hz), 6.43 (2H, d, J = 2.2 Hz), 6.27 (1H, t, J = 2.2 Hz), 5.48 (1H, bs), 5.12 (1H, d, J = 1.8 Hz), 4.32 (1H, dd, J = 8.6, 4.3 Hz), 3.79 (3H, s), 3.67 (6H, s), 3.41−3.37 (2H, m), 2.35−2.26 (1H, m), 2.06−1.98 (1H, m), 1.90−1.88 (2H, m), 1.41 (9H, s). ^{13}C NMR ($CDCl_3$, 25 °C, 125 MHz) (two conformers): δ 171.68, 171.48, 161.03, 160.76, 160.45, 160.38, 159.51, 159.27, 153.81, 152.86, 137.80, 137.76, 127.2, 126.9, 126.96, 126.83, 126.41, 126.08, 113.96, 113.73,

dx.doi.org/10.1021/jm201344a | *J. Med. Chem.* 2012, 55, 2112−2124

96.39, 96.28, 95.68, 95.58, 82.23, 82.11, 79.50, 79.38, 62.90, 57.87, 54.66, 45.94, 45.68, 30.30, 29.17, 27.74, 23.81, 23.0. ESI-MS (m/z): 549 ($M + Na^+$).

34b. Diastereoisomer with R_f = 0.32; on silica gel TLC plate, two runs, eluent *n*-hexane/*tert*-butyl methyl ether, 4:6. $[\alpha]_D$ −5.94 (*c* 30 mg/10 mL). ^1H NMR (DMSO-d_6, 80 °C, 500 MHz) δ: 7.38 (2H, d, J = 8.4 Hz), 6.98 (2H, d, J =8.4 Hz), 6.43 (2H, d, J = 2.2 Hz), 6.27 (1H, t, J = 2.2 Hz), 5.48 (1H, bs), 5.12 (1H, d, J = 1.7 Hz), 4.35 (1H, dd, J = 8.5, 4.3 Hz), 3.79 (3H, s), 3.67 (6H, s), 3.43−3.36 (1H, m), 2.35−2.26 (1H, m), 2.06−1.99 (1H, m), 1.83−1.86 (2H, m), 1.39 (9H, s). ^{13}C NMR ($CDCl_3$, 25 °C, 125 MHz) (two conformers): δ 171.35, 171.19, 161.10, 160.80, 160.42, 159.51, 159.29, 153.80, 152.86, 137.86, 137.73, 127.19, 126.89, 126.35, 126.13, 113.97, 113.82, 96.34, 95.52, 82.08, 79.63, 79.36, 62.71, 62.66, 58.39, 58.07, 54.66, 45.92, 45.65, 30.36, 29.32, 27.74, 23.92, 22.99. ESI-MS (m/z): 549 ($M + Na^+$).

Hydrazine dichloride (0.53 g, 5 mmol) was added to a stirred solution of **34b** (0.33 g, 0.63 mmol) in methanol (30 mL) at 0 °C and under nitrogen. TEA (1.0 g, 1.4 mL, 10 mmol) was added dropwise. The mixture was allowed to reach room temperature and then refluxed for 4 h. The solvent was removed at reduced pressure, and the residue was treated with a saturated solution of $KHSO_4$ and extracted with ethyl acetate (3 × 30 mL). The combined organic phases were washed with a saturated solution of $NaHCO_3$, brine and then dried over Na_2SO_4. The solvent was removed at reduced pressure, and the crude residue was purified by flash chromatography (silica gel; eluent *n*-hexane/ethyl acetate, 1:1). Compound (−)-**20** (111 mg, 0.34 mmol, 54% yield) was obtained as a white amorphous solid. $[\alpha]_D$ −29.64 ($CHCl_3$, *c* 111 mg/10 mL). The protocol reported above, applied to **34a**, afforded the enatiomer (+)-**20** (81 mg, 52% yield); $[\alpha]_D$ +29.10 ($CHCl_3$, *c* 101 mg/5 mL). The enantiomeric purity of (+)-**20** and (−)-**20** was verified via chiral HPLC by comparison with the racemic mixture and was 97% .

Resolution of (±)-*trans*-21. Compounds (−)-*trans*-**21** and (+)-*trans*-**21** were obtained following the same methodology described for the isomers **20**. The crude mixture of **35a** and **35b** was obtained with 35% yield. The mixture was purified by gradient flash chromatography (silica gel; eluent *n*-hexane/*tert*-butyl methyl ether, 1:1). Compounds **35a** and **35b** were obtained as a white amorphous solid. The two diastereoisomers were then separated by gradient flash chromatography (silica gel) using *n*-hexane/ethyl acetate as eluent (*n*-hexane/ethyl acetate ratio from 8/2 to 1/1).

35a. Diastereoisomer with R_f = 0.66 on silica gel TLC plate; (two runs, eluent, *n*-hexane/ethyl acetate, 1/1). $[\alpha]_D$ −5.83 (MeOH, *c* 120 mg/10 mL). ^1H NMR (DMSO-d_6, 80 °C, 500 MHz) δ: 7.38 (1H, dd, J = 8.4, 1.5 Hz), 7.23−7.14 (2H, m), 6.57 (2H, s), 5.52 (1H, bs), 5.18 (1H, d, J = 1.5), 4.45 (1H, dd, J = 8.76, 3.83 Hz), 4.33 (1H, dd, J = 8.61, 4.22 Hz), 3.80 (3H, s), 3.68 (6H, s), 3.64 (3H, s), 3.44−3.37 (4H, m), 2.36−2.29 (2H, m), 2.17−2.10 (1H, m), 2.00−1.87 (5H, m), 1.41 (18H, s). ^{13}C NMR ($CDCl_3$, 25 °C, 125 MHz) (two conformers): δ 172.32 and 172.29, 171.08 and 170.84, 161.53 and 161.45, 154.08 and 153.97, 153.62, 153.30 and 153.17, 151.65 and 151.57, 139.63 and 139.50, 134.91 and 134.87, 132.71, 128.27 and 128.17, 126.30 and 126.22, 121.78, 113.92 and 113.85, 95.94, 82.36 and 82.31, 79.72 and 79.68, 62.21 and 62.07, 60.55, 58.84 and 58.76, 56.51 and 56.21, 46.85 and 46.67, 38.71, 30.91 and 30.87, 29.94, and 29.82, 28.53 and 28.38, 24.54 and 24.34, 23.69, and 23.55. EI-MS (m/z): 769 (M^+).

35b. Diastereoisomer with R_f = 0.57; on silica gel TLC plate (two runs, eluent *n*-hexane/ethyl acetate, 1/1). $[\alpha]_D$ −56.12 (MeOH, *c* 131 mg/10 mL). ^1H NMR (DMSO-d_6, 80 °C, 500 MHz) δ: 7.38 (1H, d, J = 7.9 Hz), 7.20 (1H, d, J = 7.9 Hz), 7.16 (1H, bs), 6.56 (2H, s), 5.52 (1H, bs), 5.18 (1H, d, J = 1.5), 4.45 (1H, dd, J = 8.0, 3.91 Hz), 4.34 (1H, dd, J = 8.0, 4.27 Hz), 3.80 (3H, s), 3.66 (6H, s), 3.63 (3H, s), 3.44−3.37 (4H, m), 2.36−2.31 (2H, m), 2.17−2.15 (1H, m), 2.04−2.00 (1H, m), 1.93−1.88 (4H, m), 1.37 (18H, s). ^{13}C NMR ($CDCl_3$, 25 °C, 125 MHz) (two conformers) δ: 172.38 and 171.99, 171.10 and 170.88, 161.46 and 161.37, 154.03 and 153.97, 153.71, 153.31 and 153.21, 151.65 and 151.59, 139.65 and 139.49, 134.89, 132.74 and 132.66, 128.28 and 128.17, 126.46 and 126.14, 122.42 and 122.26, 121.73 and 121.62, 113.79, 96.03 and 95.83, 82.29 and 82.05, 79.66 and 79.37, 62.38 and 61.93, 60.54, 58.87 and 58.63, 56.46 and 56.23, 46.84 and 46.44, 38.79, 30.92 and 30.74, 29.97 and 29.77, 28.49 and 28.17, 24.48 and 24.37, 23.64, and 23.52. EI-MS (m/z): 769 (M^+).

The diastereoisomers **35a** and **35b** were separately treated with hydrazine dihydrochloride and purified by flash chromatography (silica gel; eluent *n*-hexane/ethyl acetate, 1:1). The compound (+)-**21** (83.5% yield) was obtained as a colorless amorphous solid. $[\alpha]_D$ +16.20 ($CHCl_3$, *c* 75 mg/5 mL). The protocol reported above, applied to **35b**, afforded the enatiomer (−)-**21** (58% yield). $[\alpha]_D$ −15.43 ($CHCl_3$, *c* 97 mg/10 mL). The enantiomeric purity of (+)-**21** and (−)-**21** was verified via chiral HPLC by comparison with the racemic mixture and was 98%.

(±)-3,4-*trans*-4-(3,5-Dimethoxyphenyl)-3-hydroxy-1-(4-methoxyphenyl)azetidin-2-one ((±)-*trans*-24) and (±)3,4-*cis*-4-(3,5-Dimethoxyphenyl)-3-hydroxy-1-(4-methoxyphenyl)-azetidin-2-one ((±)-*cis*-24). Compounds *trans*-**24** and *cis*-**24** were prepared following the method described in procedure a. Starting from imine **16** (0.87 g, 2.2 mmol), (±)-*trans*-**24** (100 mg) and (±)-*cis*-**24** (390 mg) were obtained with 14% and 55% yields, respectively. The intermediate mixture of *trans*-**22** and *cis*-**22** was obtained with 18% yield.

(±)-*trans*-**22**. ^1H NMR ($CDCl_3$): δ 7.28 (2H, d, J = 8.9 Hz), 6.79 (2H, J = 8.9 Hz), 6.45−6.40 (3H, m), 5.40 (1H, d, J = 1.2 Hz), 4.82 (1H, d, J = 1.2 Hz), 3.73 (9H,s), 2.16 (3H, s). ESI-MS (m/z): 371 (M^+).

(±)-*cis*-**22**. ^1H NMR ($CDCl_3$): δ 7.28 (2H, J = 8.9 Hz), 6.79 (2H, J = 8.9 Hz), 6.46−6.41 (3H, m), 5.94 (1H, d, J = 4.9 Hz), 5.23 (1H, d, J = 4.9 Hz), 3.73 (9H, s), 1.76 (3H, s). ^{13}C NMR (CDCl3): δ 169.88, 161.96, 161.51, 157.30, 135.60, 131.04, 119.54, 119.41, 115.05, 106.49, 101.30, 76.82, 62.13, 56.02, 20.63. ESI-MS: m/z 371 (M^+).

(±)-*trans*-**24**. ^1H NMR ($CDCl_3$): δ 7.23 (2H, d, J = 9 Hz), 6.77 (2H, d, J = 9 Hz), 6.48 (2H, d, J = 2.2 Hz), 6.42 (1H, tr, J = 2.2 Hz), 4.78 (1H, d, J = 1.5 Hz), 4.73 (1H, d, J = 1.5 Hz), 3.77 (6H, s), 3.76 (3H,s). ^{13}C NMR ($CDCl_3$): δ 165.86, 161.66, 156.37, 138.77, 129.69, 119.13, 114.54, 104.20, 100.60, 83.83, 65.87, 55.60. ESI-MS: m/z 329 (M^+).

(±)-*cis*-**24**. ^1H NMR ($CDCl_3$): δ 7.29 (2H, d, J = 8.8 Hz), 6.80 (2H, d, J = 8.8 Hz), 6.48 (3H, s), 5.16 (2H, s), 3.76 (9H, s). ^{13}C NMR ($CDCl_3$): δ 165.94, 161.62, 156.67, 135.97, 130.69, 119.03, 114.58, 105.60, 100.73, 77.40, 62.70, 55.59. ESI-MS: m/z 329 (M^+).

(±)-3,4-*trans*-3-Hydroxy-1-(3- hydroxy-4-methoxyphenyl)-4-(3,4,5-trimethoxyphenyl)azetidin-2-one ((±)-*trans*-25) and (±)-3,4-*cis*-3-Hydroxy-1-(3-hydroxy-4-methoxyphenyl)-4-(3,4,5-trimethoxyphenyl)azetidin-2-one ((±)-*cis*-25). Compounds (±)-*trans*-**25** and (±)-*cis*-**25** were prepared following the method described in procedure a. Starting from imine **17** (0.2 g, 0.38 mmol), compounds (±)-*trans*-**25** (47 mg) and (±)-*cis*-**25** (61 mg) were obtained with 33% and 43% yields, respectively.

(±)-*trans*-**23**. ^1H NMR ($CDCl_3$): δ 7.23 (1H, d, J = 2.4 Hz), 7.12 (1H, dd, J_1 = 8.9 Hz, J_2 = 2.4), 6.86 (1H, d, J = 8.9 Hz), 6.53 (2H, s), 5.40 (1H, d, J = 1.4 Hz), 4.86 (2H, s), 4.82 (1H, d, J = 1.4 Hz), 3.85 (3H, s), 3.82 (6H,s), 3.78 (3H, s), 2.20 (3H, s), 2.18 (3H, s). ^{13}C NMR ($CDCl_3$): δ 170.06, 169.65, 165.69, 161.39, 153.89, 148.13, 139.16, 138.45, 130.32, 115.98, 112.97, 112.82, 103.22, 82.54, 64.12, 60.82, 60.25, 56.20, 20.49. ESI-MS: m/z 540 ($M + Na^+$).

(±)-*cis*-**23**. ^1H NMR ($CDCl_3$): δ 7.26 (1H, d, J = 2.5 Hz), 7.14 (1H, dd, J_1 = 8.9 Hz, J_2 = 2.5 Hz), 6.88 (1H, d, J = 8.9 Hz), 6.48 (2H, s), 5.94 (1H, d, J = 4.9 Hz), 5.25 (1H, d, J = 4.9 Hz), 4.87 (2H, s), 3.88 (3H, s), 3.80 (6H, s), 3.79 (3H, s), 2.18 (3H), 1.76 (3H). ^{13}C NMR ($CDCl_3$): δ 170.22, 169.35, 165.84, 161.74, 153.49, 148.38, 139.37, 138.45, 130.46, 127.68, 116.08, 113.02, 104.98, 76.68, 62.04, 61.43, 60.41, 56.40, 20.56, 20.15. ESI-MS: m/z 540 ($M + Na^+$).

(±)-*trans*-**25**. ^1H NMR ($CDCl_3$): δ 6.90 (1H, d, J = 2.4 Hz), 6.83 (1H, dd, J_1 = 2.4, 8.6 Hz), 6.71 (1H, d, J = 8.6 Hz), 6.52 (2H, s), 5.31 (1H, s), 4.76 (1H, d, J = 1.6 Hz), 4.70 (1H, d, J = 1.6 Hz), 3.86 (3H, s), 3.85 (3H, s), 3.82 (6H, s). ^{13}C NMR ($CDCl_3$): δ 166.28, 154.07, 146.05, 131.84, 131.22, 111.05, 109.69, 105.05, 102.98, 83.90, 66.18, 61.03, 56.40. ESI-MS: m/z 375 (M^+).

(±)-*cis*-**25**. ^1H NMR (400 MHz, $CDCl_3$): δ 6.98 (1H, d, J = 8.0 Hz), 6.95 (1H, s), 6.79 (1H, d, J = 8.0 Hz), 6.53 (2H, s), 5.69 (1H, s),

5.19−5.15 (2H, m), 3.88 (6H, s), 3.85 (6H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ 156.00, 147.00, 129.80, 111.68, 111.09, 105.28, 104.71, 63.29, 61.52, 56.89. ESI-MS: *m/z* 375 (M^+).

(±)-3,4-*trans*-3-Amino-1-(3,5-dimethoxyphenyl)-4-(4-methoxyphenyl)azetidin-2-one ((±)-*trans*-28) and (±)-3,4-*cis*-3-Amino-1-(3,5-dimethoxyphenyl)-4-(4-methoxyphenyl)-azetidin-2-one ((±)-*cis*-28) (Traces). A solution of phthalylglycyl chloride (1.36 g, 6.08 mmol) in anhydrous methylene chloride (3 mL) was added dropwise at 0 °C under nitrogen to a stirred solution of imine **14** (0.375 g, 1.38 mmol) and TEA (1.23 g, 1.7 mL, 12.6 mmol) in anhydrous methylene chloride (7 mL). The solution was maintained at 0 °C for 1 h and then allowed to reach room temperature and stirred for 24 h. HCl (1 N) was added (40 mL), and the two phases were stirred for 40 min. The aqueuos phase was separated and extracted twice with dichloromethane. Organic phases were washed with a saturated solution of $NaHCO_3$ dried with sodium sulfate, filtered, and concentrated under reduced pressure, affording a brown oil which was purified by flash column chromatography (silica gel; eluent gradient of *n*-hexane/ethyl acetate from 7/3 to 4/6). The isomer (±)-*trans*-**26** only was isolated (0.289 g, 0.62 mmol, 46% yield). Compound (±)-*cis*-**26** was formed only in traces from the reaction. (±)-*trans*-**26**: ^{1}H NMR ($CDCl_3$) δ 7.9−7.7 (4H, m), 7.32 (2H, *J* = 8.8 Hz), 6.82 (2H, *J* = 8.8 Hz), 6.50 (2H, d, *J* = 2.2 Hz), 6.44 (1H, tr, *J* = 2.2 Hz), 5.29 (1H, d, *J* = 2.7 Hz), 5.26 (1H, d, *J* = 2.7 Hz), 3.76 (9H, s). ^{13}C NMR ($CDCl_3$): δ 166.99, 161.80, 156.74, 138.57, 134.73, 131.93, 130.95, 123.98, 119.25, 114.57, 104.32, 100.94, 62.82, 61.65, 55.62. ESI-MS: *m/z* 481 (M + Na^+).

Hydrazine dihydrochloride (0.30 g, 2.8 mmol) was added, at 0 °C and under nitrogen to a stirred suspension of (±)-*trans*-**26** (0.6 mg, 1.3 mmol) in methanol (10 mL). TEA (1.6 g, 2.2 mL, 15.8 mmol) was added dropwise. The mixture was allowed to reach room temperature and then warmed at 50 °C for 5 h. The solvent was removed at reduced pressure, and the residue was treated with 1 N HCl (40 mL) and extracted with dichloromethane (3 × 10 mL). The aqueous phase was made alkaline by 3 N NaOH and extracted with dichloromethane (3 × 10 mL). The organic phase was dried (Na_2SO_4), and the solvent was removed at reduced pressure. A solid was obtained, which was purified by flash chromatography (silica gel; eluent *n*-hexane/ethyl acetate, 2/8) to afford the stereoisomer (±)-*trans*-**28** (0.36 g, 1.1 mmol, 85% yield) as a yellow solid. ^{1}H NMR ($CDCl_3$) δ 7.29 (2H, d, *J* = 8.7 Hz), 6.93 (2H, d, *J* = 8.7 Hz), 6.49 (1H, d, *J* = 2.2 Hz), 6.41 (1H, tr, *J* = 2.2 Hz), 4.64 (1H, d, *J* = 2.2 Hz), 4.10 (1H, d, *J* = 2.2 Hz), 3.75 (9H, s). ^{13}C NMR ($CDCl_3$): δ 161.65, 139.41, 118.92, 114.52, 104.02, 100.51, 69.27, 66.40, 55.59. ESI-MS: *m/z* 351 (M + Na^+).

(±)-3,4-*trans*-3-Amino-4-(3-hydroxy-4-methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one ((±)-*trans*-29). Compounds (±)-*trans*-**27** and (±)-*cis*-**27** were prepared following the procedures described for compound **26**. Starting from imine **15** (0.25 g, 0.8 mmol), the isomer *trans*-**27** only was isolated (0.34 g, 0.50 mmol 63% yield) after purification by flash column chromatography (silica gel; eluent gradient of *n*-hexane/ethyl acetate from 85/15 to 40/60). Compound *cis*-**27** was formed only in traces from the reaction. (±)-*trans*-**27**: ^{1}H NMR ($CDCl_3$) δ 8−7.6 (8H, m), 7.29 (1H, dd, J_1 = 8.4 Hz, J_2 = 2.2 Hz), 7.19 (1H, d, *J* = 2.2 Hz), 7.00 (1H, d, *J* = 8.4 Hz), 6.57 (2H, s), 5.28 (2H, s), 4.71 (2H, s), 3.85 (3H, s), 3.76 (3H, s), 3.70 (6H, s). ^{13}C NMR ($CDCl_3$): δ 167.46, 166.96, 165.41, 162.03, 140.09, 134.79, 134.53, 133.40, 132.15, 131.86, 128.37, 126.40, 125.08, 124.27, 123.87, 121.47, 113.64, 95.50, 67.78, 61.07, 56.24, 38.89. ESI-MS: *m/z* 691 (M^+).

Compound (±)-*trans*-**27** was treated with hydrazine dihydrochloride and affords after purification by flash chromatography (silica gel; eluent *n*-hexane/ethyl acetate, 2/8) the stereoisomer (±)-*trans*-**29** (0.048 g, 0.13 mmol, 81% yield) as a yellow amorphous solid. ^{1}H NMR ($CDCl_3$): δ 6.92 (1H, s), 6.89 (2H, s), 6.54 (2H, s), 4.54 (1H, d, *J* = 2.2 Hz), 4.05 (1H, d, *J* = 2.2 Hz), 3.88 (3H, s), 3.76 (3H, s), 3.71 (6H,s). ^{13}C NMR ($CDCl_3$): δ 168.05, 153.65, 147.11, 146.51, 134.80, 133.80, 130.14, 118.14, 117.99, 112.33, 111.22, 95.38, 69.70, 66.70, 61.09, 56.22. ESI-MS: *m/z* 374 (M^+).

(±)-3,4-*trans*-3-Amino-4-(3,5-dimethoxyphenyl)-1-(4-methoxyphenyl)azetidin-2-one ((±)-*trans*-32) and (±)-3,4-*cis*-3-Amino-4-(3,5-dimethoxyphenyl)-1-(4-methoxyphenyl)-azetidin-2-one ((±)-*cis*-32). Compounds (±)-*trans*-**30** and (±)-*cis*-**30** were prepared following the procedures described for compound **26**. Starting from imine **16** (1.74 g, 6.42 mmol), the mixture of isomers *trans*-**30** and *cis*-**30** were isolated (0.55 g, 1.20 mmol 19% yield) after purification by flash column chromatography (silica gel; eluent *n*-hexane/ethyl acetate, 1:1).

(±)-*trans*-**30**. ^{1}H NMR ($CDCl_3$): δ 7.91−7.89 (2H, m), 7.79−7.77 (2H, m), 7.32 (2H, d, *J* = 8.8 Hz), 6.84 (2H, d, *J* = 8.8 Hz), 6.51 (2H, d, *J* = 2.4 Hz), 6.45 (1H, tr, *J* = 2.4 Hz), 5.30 (1H, d, *J* = 2.4 Hz), 5.26 (1H, d, *J* = 2.4 Hz), 3.78 (9H, s). ^{13}C NMR ($CDCl_3$): δ 166.99, 161.80, 156.74, 138.57, 134.73, 134.46, 131.93, 130.95, 123.99, 123.64, 118.84, 115.49, 104.32, 62.87, 61.66, 55.62. ESI-MS: *m/z* 481 (M + Na^+).

(±)-*cis*-**30**. ^{1}H NMR ($CDCl_3$): δ 7.9−7.4 (4H, m), 7.41 (2H, d, *J* = 6.8 Hz), 7.00 (2H, d, *J* = 6.8 Hz), 6.45 (2H, d, *J* = 2.2 Hz), 6.20 (1H, tr, *J* = 2.2 Hz), 5.63 (1H, d, *J* = 5.5 Hz), 5.35 (1H, d, *J* = 5.5 Hz), 3.78 (3H,s), 3.63 (6H, s). ^{13}C NMR ($CDCl_3$): δ 166.93, 160.90, 160.44, 156.71, 134.94, 134.73, 131.92, 131.49, 123.83, 123.63, 119.24, 114.57, 105.22, 62.18, 61.37, 55.55. ESI-MS: *m/z* 481 (M + Na^+).

Compounds (±)-*trans*-**30** and (±)-*trans*-**30** were treated with hydrazine dihydrochloride and afford after purification by flash chromatography (silica gel; eluent *n*-hexane/ethyl acetate, 2/8) the stereoisomer (±)-*trans*-**32** (7% yield) as a yellow amorphous solid and the stereoisomer (±)-*cis*-**32** (8% yield).

(±)-*trans*-**32**. ^{1}H NMR ($CDCl_3$): δ 7.30 (2H, d, *J* = 9.5 Hz), 6.80 (2H, d, *J* = 9.5 Hz), 6.48 (2H, d, *J* = 2.2 Hz), 6.42 (1H, t, *J* = 2.2 Hz), 4.55 (1H, d, *J* = 2.0 Hz), 4.06 (1H, d, *J* = 2.0 Hz), 3.76 (9H, s). ^{13}C NMR ($CDCl_3$): δ 167.50, 161.56, 156.39, 139.64, 131.15, 118.85, 114.52, 103.95, 100.42, 69.90, 66.98, 55.59. ESI-MS: *m/z* 351(M + Na^+).

(±)-*cis*-**32**. ^{1}H NMR ($CDCl_3$): δ 7.30 (2H, d, *J* = 9 Hz), 6.80 (2H, d, *J* = 9 Hz), 6.42 (1H, t, *J* = 2.2 Hz), 6.39 (2H, d, *J* = 2.2 Hz), 5.14 (1H, d, *J* = 5.4 Hz), 4.59 (1H, d, *J* = 5.4 Hz), 3.76 (9H, s). ^{13}C NMR ($CDCl_3$): δ 167.46, 161.67, 156.37, 137.22, 131.14, 118.82, 114.51, 105.22, 103.95, 100.25, 69.91, 66.96, 63.99, 62.59, 55.59. ESI-MS: *m/z* 351 (M + Na^+).

(±)-3,4-*trans*-3-Amino-4-(3-hydroxy-4-methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one ((±)-*trans*-33) and (±)-3,4-*cis*-3-Amino-4-(3-hydroxy-4-methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one ((±)-*cis*-33). Compounds (±)-*trans*-**31** and (±)-*cis*-**31** were prepared following the procedures described for compound **26**. Starting from imine **17** (0.324 g, 1.02 mmol), the mixture of isomers (±)-*trans*-**27** and (±)-*cis*-**27** was isolated (0.48 g, 0.7 mmol 68% yield) after purification by flash column chromatography (silica gel; eluent *n*-hexane/ethyl acetate, 45:55).

(±)-*trans*-**31**. ^{1}H NMR ($CDCl_3$): δ 8.0−7.8 (8H, m), 7.27 (1H, d, *J* = 2.6 Hz), 7.16 (1H, dd, J_1 = 2.6 Hz, J_2 = 8.9 Hz), 6.88 (1H, d, *J* = 8.9 Hz), 6.56 (2H, s), 5.29 (1H, d, *J* = 2.6 Hz), 5.25 (1H, d, *J* = 2.6 Hz), 4.71 (2H, s) 3.86 (3H, s), 3.82 (6H,s), 3.80 (3H, s). ^{13}C NMR ($CDCl_3$): δ 167.42, 166.97, 165.34, 161.98, 148.23, 139.77, 134.80, 134.45, 132.20, 131.89, 131.25, 130.85, 124.05, 123.86, 116.36, 113.12, 103.16, 63.12, 62.10, 61.03, 56.43, 38.87. ESI-MS: *m/z* 691 (M^+).

(±)-*cis*-**31**. ^{1}H NMR ($CDCl_3$): δ 7.91−7.75 (8H, m), 7.69 (1H, d, *J* = 2.3 Hz), 7.38 (1H, dd, J_1 = 2.3 Hz, J_2 = 8.9 Hz), 6.94 (1H, d, *J* = 8.9 Hz), 6.49 (2H, s), 5.63 (1H, d, *J* = 5.4 Hz), 5.34 (1H, d, *J* = 5.4 Hz), 4.72 (2H, s), 3.84 (3H, s), 3.72 (6H, s), 3.65 (3H,s). ESI-MS: *m/z* 691 (M^+).

Compounds (±)-*trans*-**33** and (±)-*cis*-**33** were obtained following the procedures described for compound **26**. Starting from a mixture of compounds (±)-*trans*-**31** and (±)-*cis*-**31** (179 mg, 0.26 mmol), isomers (±)-*trans*-**33** (33% yield) and (±)-*cis*-**33** (22% yield) were isolated after purification by flash column chromatography (silica gel; eluent hexane/ethyl acetate = 2:8).

(±)-*trans*-**33**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.94 (1H, d, *J* = 2.4 Hz), 6.79 (1H, dd, J_1 = 8.7 Hz, J_2 = 2.4 Hz), 6.72 (1H, d, *J* = 8.7 Hz), 6.53 (2H, s), 4.54 (1H, d, *J* = 2.0 Hz), 4.04 (1H, d, *J* = 2.0 Hz), 3.85 (3H, s), 3.82 (3H, s), 3.80 (6H, s). ^{13}C NMR (100 MHz; $CDCl_3$): δ

168.39, 154.53, 146.73, 144.33, 133.23, 132.16, 111.77, 109.75, 105.45, 103.29, 70.37, 67.53, 61.48, 56.84. ESI-MS: m/z 374 (M^+).

(±)-*cis*-**33**. ^{1}H NMR (400 MHz; $CDCl_3$): δ 6.98−6.94 (2H, m), 6.77 (1H, d, J = 8.4 Hz), 6.46 (2H, s), 5.13 (1H, d, J = 5.2 Hz), 4.58 (1H, d, J = 5.2 Hz), 3.87 (6H, s), 3.82 (6H, s). ^{13}C NMR (100 MHz; $CDCl_3$): δ 168.29, 154.59, 146.64, 144.17, 138.69, 132.20, 130.60, 111.74, 109.87, 105.30, 104.48, 67.64, 64.36, 61.47, 56.85. ESI-MS: m/z 374 (M^+).

Biology: Materials and Methods. Reagents and Antibodies. Anti-AMPK, anti-phospho-AMPK (Thr172), anti-caspase-3, anti-PARP, anti-Raptor, anti-phospho-Raptor (Thr792), and anti-vinculin for Western blotting analysis were purchased from Cell Signaling Technologies (Beverly, MA). Anti-fibrillarin antibody, used as nuclear extract control, was purchased from EnCor Biotechnology (Gainesville, FL). Dulbecco's modified Eagle's medium (DMEM), Eagle's minimal essential medium medium (EMEM), F12 medium, Leibovitz medium, fetal bovine serum (FBS), L-glutamine, penicillin, and streptomycin were obtained from Lonza (Basel, Switzerland). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Cell Cultures. HuTu-80 cells and HeLa cells were cultured using DMEM supplemented with 10% (v/v) FBS, 4 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. FHs74 cells were cultured using DMEM supplemented with 10% (v/v) FBS, 4 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 30 ng/mL EGF, and 10 μg/mL insulin. CCD841CoN cells and MCF-7 cells were cultured using EMEM/NEAA supplemented with 10% (v/v) FBS, 4 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. T84 cells were cultured using DMEM/F12 medium supplemented with 5% (v/v) FBS, 4 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. SKNBE cells were cultured using EMEM/F12 medium supplemented with 10% (v/v) FBS, 4 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. All these cell lines were maintained at 37 °C in a humidified 5% CO_2 incubator. SW48, SW480, and SW620 cells were cultured using Leibovitz's medium supplemented with 10% (v/v) FBS, 4 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. These cell lines were maintained at 37 °C in a humidified incubator.

Subcellular Fractionation and Total Protein Extraction. To detect p53 levels and phosphorylation, nuclear extracts were prepared using the NE-PER kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer's protocol. To analyze all the other proteins, total cell extracts were prepared using RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP40, 0.1% SDS) plus 1 mM PMSF (phenylmethanesulfonyl fluoride), protease inhibitor cocktail (Roche, Indianapolis, IN), and phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO).

Flow Cytofluorimetric Analysis. For flow cytometric analysis, cells were trypsinized, washed with PBS, and fixed in 75% ethanol at 4 °C. Samples were stained with propidium iodide (Sigma-Aldrich, St. Louis, MO) to analyze DNA content using a BD Biosciences FACScan (Becton-Dickinson, San Jose, CA).

Growth Inhibition Assay. The antiproliferative activity of the azetidinone derivatives was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded in 96-well plates. After 24 h of incubation in the appropriate medium, cells were treated with different concentrations of each compound for 48−72 h, as indicated in the figure captions. Afterward, the MTT solution was added, and then plates were incubated for 2 h at 37 °C. The purple formazan crystals were solubilized. and the plates were read on a model 550 microplate reader (BioRad Laboratories, Hercules, CA) at 570 nm. The IC_{50} values were obtained using the software GraphPad Prism (San Diego, CA) and were defined as the concentration of drug causing a 50% inhibition in absorbance compared to control cells. Assays were performed in triplicate in three independent experiments.

Tubulin Polymerization Assay. The effect of compounds on the assembly of tubulin was determined spectrophotometrically by monitoring the change in turbidity. Lyophilized purified porcine brain tubulin (Cytoskeleton, Denver, CO) was resuspended in ice-cold assembly buffer (80 mM PIPES, pH 6.9, 1 mM $MgCl_2$, 2 mM EGTA, 10% glycerol) at 2.5 mg/mL and mixed with 1 mM GTP and varying concentrations of the compounds. DMSO (0.2% v/v) was used as a vehicle control. Tubulin assembly was monitored at 340 nm at 37 °C in a Jasco V-530 spectrophotometer (Jasco Europe, Italy). The IC_{50} values were obtained using the software GraphPad Prism (San Diego, CA) and were defined as the compound concentration that inhibited the extent of assembly by 50% after a 30 min incubation. Assays were performed in triplicate in two independent experiments.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figure S1 showing that the new azetidinone compounds exhibit antiproliferative activity on HeLa cells and Figure S2 showing that the new β-lactams induce apoptotic cell death in cancer cells. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*For R.P.: phone, +39-0250314118; e-mail, roberto.pagliarin@unimi.it. For P.C.: phone, +39-0264483521; e-mail, paola.coccetti@unimib.it.

Present Address

⊥IFOM-IEO Campus, Via Adamello, 16, 20139 Milan, Italy.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Prof. Lilia Alberghina for helpful discussions and support and Neil Campbell for language editing. This work was supported by a grant to P.C. (FAR 2008), to P.F. (FAR 2010), to M.F. (Oncosuisse), and to F.O. (PRIN 2007, Grant 2007K29W5J and PRIN 2009, Grant 2009KFMP7Z). The patent in ref 15 was supported by UNIMITT of University of Milan, Italy, and by University of Milano-Bicocca, Italy.

■ DEDICATION

†We dedicate this work to the memory of our friend Alessandro Gori, who believed and hoped in scientific research and whom we thank for his encouragement.

■ ABBREVIATIONS USED

CA-4, combretastatin A-4; AMP, adenosine monophosphate; AMPK, adenosine monophosphate activated protein kinase; ATP, adenosine 5′-triphosphate; CRC, colorectal cancer; DIPEA, *N*,*N*-diisopropylethylamine; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; Ft, ftalyl; EGF, epidermal growth factor; EGTA, ethylene glycol tetraacetic acid; GTP, guanidine triphosphate; HBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; IC_{50}, 50% inhibitory concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMR, nuclear magnetic resonance; PARP, poly ADP-ribose polymerase; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PMSF, phenylmethanesulfonyl fluoride; SBA, small bowel adenocarcinoma; SDS, sodium dodecyl sulfate; TEA, triethylamine; TMS, tetramethylsilane

■ REFERENCES

(1) Yue, Q.-X.; Liu, X; Guo, D.-A. Microtubule-binding natural products for cancer therapy. *Planta Med.* **2010**, *76*, 1037−1043.

(2) Lin, C. M.; Ho, H. H.; Pettit, G. R.; Hamel, E. Antimitotic natural products combretastatin A-4 and combretastatin A-2: studies on the

mechanism of their inhibition of the binding of colchicine to tubulin. *Biochemistry* **1989**, *28*, 6984−6991.
(3) Lippert, J. W. Vascular disrupting agents. *Bioorg. Med. Chem.* **2007**, *15*, 605−615.
(4) Nam, N.-H. Combretastatin A-4 analogues as antimitotic antitumor agents. *Curr. Med. Chem.* **2003**, *10*, 1697−1722.
(5) West, C. M. L.; Price, P. Combretastatin A4 phosphate. *Anti-Cancer Drugs* **2004**, *15*, 179−87.
(6) www.clinicaltrials.gov.
(7) Tron, G. C.; Pirali, T.; Sorba, G.; Pagliai, F.; Busacca, S.; Genazzani, A. A. Medicinal chemistry of combretastatin A4: present and future directions. *J. Med. Chem.* **2006**, *49*, 3033−3044.
(8) Wang, L.; Woods, K. W.; Li, Q.; Barr, K. J.; McCroskey, R. W.; Hannick, S. M.; Gherke, L.; Credo, R. B.; Hui, Y.-H.; Marsh, K.; Warner, R.; Lee, J. Y.; Zielinski-Mozng, N.; Frost, D.; Rosenberg, S. H.; Sham, H. L. Potent, orally active heterocycle-based combretastatin A-4 analogues: synthesis, structure−activity relationship, pharmacokinetics, and in vivo antitumor activity evaluation. *J. Med. Chem.* **2002**, *45*, 1697−1711.
(9) O'Boyle, N. M.; Carr, M.; Greene, L. M.; Bergin, O.; Nathwani, S. M.; McCabe, T.; Lloyd, D. G.; Zisterer, D. M.; Meegan, M. J. Synthesis and evaluation of azetidinone analogues of combretastatin A-4 as tubulin targeting agents. *J. Med. Chem.* **2010**, *53*, 8569−8584.
(10) Banik, B. K.; Banik, I.; Becker, F. F. Novel anticancer β-lactams. *Top Heterocycl. Chem* **2010**, *22*, 349−373.
(11) Smith, D. M.; Kazi, A.; Smith, L.; Long, T. E.; Heldreth, B.; Turos, E.; Dou, Q. P. A novel beta-lactam antibiotic activates tumor cell apoptotic program by inducing DNA damage. *Mol. Pharmacol.* **2002**, *61*, 1348−1358.
(12) Sun, L.; Vasilevich, N. I.; Fuselier, J. A.; Hocart, S. J.; Coy, D. H. Examination of the 1,4-disubstituted azetidinone ring system as a template for combretastatin A-4 conformationally restricted analogue design. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2041−2046.
(13) O'Boyle, N. M.; Greene, L. M.; Bergin, O.; Fichet, J. B.; McCabe, T.; Lloyd, D. G.; Zisterer, D. M.; Meegan, M. J. Synthesis, evaluation and structural studies of antiproliferative tubulin-targeting azetidin-2-ones. *Bioorg. Med. Chem.* **2011**, *19*, 2306−2325.
(14) Carr, M.; Greene, L. M.; Knox, A. J.; Lloyd, D. G.; Zisterer, D. M.; Meegan, M. J. Lead identification of conformationally restricted β-lactam type combretastatin analogues: synthesis, antiproliferative activity and tubulin targeting effects. *Eur. J. Med. Chem.* **2010**, *45* (12), 5752−5766.
(15) Pagliarin, R; Orsini, F.; Montano, G.; Tripodi, F.; Coccetti, P.; Fusi, P. Patent MI2011A001463, 2011.
(16) Water solubility for (±)-*trans*-**20** is 95 μM, and water solubility for (±)-*trans*-**21** is 1700 μM (see Experimental Section).
(17) Petrovski, G.; Gurusamy, N.; Das, D. K. Resveratrol in cardiovascular health and disease. *Ann. N.Y. Acad. Sci.* **2011**, *1215*, 22−33.
(18) Kondratyuk, T. P.; Park, E.-J.; Marler, L. E.; Ahn, S.; Yuan, Y.; Choi, Y.; Yu, R.; van Breemen, R. B.; Sun, B.; Hoshino, J.; Cushman, M.; Jermihov, K. C.; Mesecar, A. D.; Grubbs, C. J.; Pezzuto, J. M. Resveratrol derivatives as promising chemopreventive agents with improved potency and selectivity. *Mol. Nutr. Food Res.* **2011**, *55*, 1249−1265.
(19) Zhang, F.; Sun, C.; Wu, J.; He, C.; Ge, X.; Huang, W.; Zou, Y.; Chen, X.; Qi, W.; Zhai, Q. Combretastatin A-4 activates AMP-activated protein kinase and improves glucose metabolism in db/db mice. *Pharmacol. Res.* **2008**, *57*, 318−323.
(20) Coccetti, P.; Montano, G.; Lombardo, A.; Tripodi, F.; Orsini, F.; Pagliarin, R. Synthesis and biological evaluation of combretastatin analogs as cell cycle inhibitors of the G1 to S transition in *Saccharomyces cerevisiae*. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 2780−2784.
(21) Hardie, D. G.; Carling, D.; Carlson, M. The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? *Annu. Rev. Biochem.* **1998**, *67*, 821−855.
(22) Shackelford, D. B.; Shaw, R. J. The LKB1-AMPK pathway: metabolism and growth control in tumour suppression. *Nat. Rev. Cancer* **2009**, *9*, 563−575.
(23) Buzzai, M.; Jones, R. G.; Amaravadi, R. K.; Lum, J. J.; DeBerardinis, R. J.; Zhao, F.; Viollet, B.; Thompson, C. B. Systemic treatment with the antidiabetic drug metformin selectively impairs p53-deficient tumor cell growth. *Cancer Res.* **2007**, *67*, 6745−6752.
(24) Zakikhani, M.; Dowling, R. J. O.; Sonenberg, N.; Pollak, M. N. The effects of adiponectin and metformin on prostate and colon neoplasia involve activation of AMP-activated protein kinase. *Cancer Prev. Res.* **2008**, *1*, 369−375.
(25) Fay, J. R.; Steele, V.; Crowell, J. A. Energy homeostasis and cancer prevention: the AMP-activated protein kinase. *Cancer Prev. Res.* **2009**, *2*, 301−309.
(26) Ji, C.; Yang, B.; Yang, Y.-L.; He, S.-H.; Miao, D.-S.; He, L.; Bi, Z.-G. Exogenous cell-permeable C6 ceramide sensitizes multiple cancer cell lines to doxorubicin-induced apoptosis by promoting AMPK activation and mTORC1 inhibition. *Oncogene* **2010**, *29*, 6557−6568.
(27) Jiao, L.; Liang, Y.; Xu, J. Origin of the relative stereoselectivity of the beta-lactam formation in the Staudinger reaction. *J. Am. Chem. Soc.* **2006**, *128*, 6060−6069.
(28) Endo, M.; Droghini, R. Synthesis of *trans*-1-*p*-methoxyphenyl-3-acetoxy-4-phenylazetidin-2-one. A key starting β-lactam for 2′-epi-taxol. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 2483−2486.
(29) Shen, C.-H.; Shee, J.-J.; Wu, J.-Y.; Lin, Y.-W.; Wu, J.-D.; Liu, Y.-W. Combretastatin A-4 inhibits cell growth and metastasis in bladder cancer cells and retards tumour growth in a murine orthotopic bladder tumour model. *Br. J. Pharmacol.* **2010**, *160*, 2008−2027.
(30) Wu, R.; Ding, W.; Liu, T.; Zhu, H.; Hu, Y.; Yang, B.; He, Q. XN05, a novel synthesized microtubule inhibitor, exhibits potent activity against human carcinoma cells in vitro. *Cancer Lett.* **2009**, *285*, 13−22.
(31) Overman, M. J.; Kopetz, S.; Wen, S.; Hoff, P. M.; Fogelman, D.; Morris, J.; Abbruzzese, J. L.; Ajani, J. A.; Wolff, R. A. Chemotherapy with 5-fluorouracil and a platinum compound improves outcomes in metastatic small bowel adenocarcinoma. *Cancer* **2008**, *113*, 2038−2045.
(32) Delaunoit, T.; Neczyporenko, F.; Limburg, P. J.; Erlichman, C. Small bowel adenocarcinoma: a rare but aggressive disease. *Clin. Colorectal Cancer* **2004**, *4*, 241−248 (discussion, 249−251).
(33) Bläker, H.; Aulmann, S.; Helmchen, B.; Otto, H. F.; Rieker, R. J.; Penzel, R. Loss of SMAD4 function in small intestinal adenocarcinomas: comparison of genetic and immunohistochemical findings. *Pathol. Res. Pract.* **2004**, *200*, 1−7.
(34) Jones, R. G.; Plas, D. R.; Kubek, S.; Buzzai, M.; Mu, J.; Xu, Y.; Birnbaum, M. J.; Thompson, C. B. AMP-activated protein kinase induces a p53-dependent metabolic checkpoint. *Mol. Cell* **2005**, *18*, 283−293.
(35) Khanal, P.; Oh, W.-K.; Yun, H. J.; Namgoong, G. M.; Ahn, S.-G.; Kwon, S.-M.; Choi, H.-K.; Choi, H. S. p-HPEA-EDA, a phenolic compound of virgin olive oil, activates AMP-activated protein kinase to inhibit carcinogenesis. *Carcinogenesis* **2011**, *32*, 545−553.
(36) Imamura, K.; Ogura, T.; Kishimoto, A.; Kaminishi, M.; Esumi, H. Cell cycle regulation via p53 phosphorylation by a 5′-AMP activated protein kinase activator, 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside, in a human hepatocellular carcinoma cell line. *Biochem. Biophys. Res. Commun.* **2001**, *287*, 562−567.
(37) Gwinn, D. M.; Shackelford, D. B.; Egan, D. F.; Mihaylova, M. M.; Mery, A.; Vasquez, D. S.; Turk, B. E.; Shaw, R. J. AMPK phosphorylation of raptor mediates a metabolic checkpoint. *Mol. Cell* **2008**, *30*, 214−226.
(38) Priebe, A.; Tan, L.; Wahl, H.; Kueck, A.; He, G.; Kwok, R.; Opipari, A.; Liu, J. R. Glucose deprivation activates AMPK and induces cell death through modulation of Akt in ovarian cancer cells. *Gynecol. Oncol.* **2011**, *122*, 389−395.
(39) Luo, J.; Manning, B. D.; Cantley, L. C. Targeting the PI3K-Akt pathway in human cancer. *Cancer Cell* **2003**, *4*, 257−262.

(40) Water solubility of combretastatin A4 is 350 μM. Lee, M.; Brockway, O.; Dandavati, A.; Tzou, S.; Sjoholm, R.; Satam, V.; Westbrook, C.; Mooberry, S. L.; Zeller, M.; Babu, B.; Lee, M. A novel class of trans-methylpyrazoline analogs of combretastatins: synthesis and in-vitro biological testing. *Eur. J. Med. Chem.* **2011**, *46*, 3099−3104.
(41) Wang, W.; Guan, K.-L. AMP-activated protein kinase and cancer. *Acta Physiol.* **2009**, *196*, 55−63.
(42) Wullschleger, S.; Loewith, R.; Hall, M. N. TOR signaling in growth and metabolism. *Cell* **2006**, *124*, 471−484.