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Biological Evaluation *in Vitro* and *in Silico* of Azetidin-2-one Derivatives as Potential Anticancer Agents

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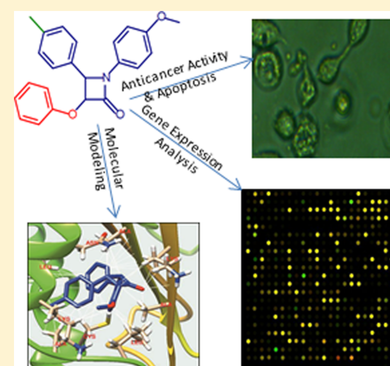
Supporting Information

ABSTRACT: Potential anticancer activity of 16 azetidin-2-one derivatives was evaluated showing that compound **6** [*N*-(*p*-methoxy-phenyl)-2-(*p*-methyl-phenyl)-3-phenoxy-azetidin-2-one] presented cytotoxic activity in SiHa cells and B16F10 cells. The caspase-3 assay in B16F10 cells displayed that azetidin-2-one derivatives induce apoptosis. Microarray and molecular analysis showed that compound **6** was involved on specific gene overexpression of cytoskeleton regulation and apoptosis due to the inhibition of some cell cycle genes. From the 16 derivatives, compound **6** showed the highest selectivity to neoplastic cells, it was an inducer of apoptosis, and according to an *in silico* analysis of chemical interactions with colchicine binding site of human α/β-tubulin, the mechanism of action could be a molecular interaction involving the amino acids outlining such binding site.



KEYWORDS: *Azetidin-2-one, anticancer, β-tubulin, apoptosis, microarray, docking*

Cancer is caused by the uncontrolled proliferation of genetically transformed cells with the capacity of metastasis and evasion of apoptosis.[1] Some chemotherapeutic agents are derived from natural sources such as taxane and vinca alkaloid products. Some alkaloid derivatives have a mechanism of action as vascular disrupting agents (VDAs) like vincristine and colchicine; these bind to tubulin inhibiting microtubule polymerization during the process of mitosis.[2] Currently, some of the most important anticancer drug projects are aimed to the rational design of drugs with specific activity against certain types of biological tissues allowing in the future the accessibility to more specific anticancer therapy,[3,4] as well as to the developing of new VDAs with tubulin affinity.[5] Derivatives of colchicine have shown promising results as VDAs, like combretastatin A-4 phosphate (CA4P),[6] Oxi4503,[7] AVE8062,[8] and ZD6126[9] allowing using them in clinical trials.

CA4P has been modified obtaining its *cis* stereoisomer through the incorporation of a β-lactam ring (azetidin-2-one).[10] Thus, an improved stability was witnessed in CA4P and consequently a considerable increased antineoplastic activity, as described by Carr et al.[11] This, in turn, supports the fact that azetidine-2-one derivatives have an antitumoral activity on cell line MCF-7 reaching an IC_{50} of 0.8 nM.[12] However, the mechanism of action of azetidine-2-one derivatives has not been completely described at the molecular level. In this work, 16 derivatives of azetidin-2-one are presented. Some of these derivatives showed high cytotoxic activity and specificity on cancer cell lines B16F10 and SiHa with respect to vincristine. Furthermore, induction of apoptosis, transcriptional analysis through a DNA microarray and molecular docking in binding sites on human tubulin are also showed.

Sixteen azetidin-2-one derivatives were obtained by Staudinger reaction of aromatic imines and an acid chloride. Compounds were grouped into three series: *N*-(4-methoxy-phenyl)-3-phenoxy-azetidin-2-one derivatives (**1**−**6**), *N*-(4-methoxy-phenyl)-3-methoxy-azetidin-2-one derivatives (**7**−**12**), and *N*-(1,3-benzothiazol)-3-phenoxy-azetidin-2-one racemate derivatives (**13**−**16**) (Figure 1, Table 1). Compounds **1**−**12** were designed following previous β-lactams derivatives using different substituents at the azetidine moiety (R3, Figure 1). These were selected according to electronic properties and solubility contributions.[13,14] The benzothiazole group has been associated with antitumoral agents; therefore, this moiety was incorporated in our design to enhance the activity.[15,16] The

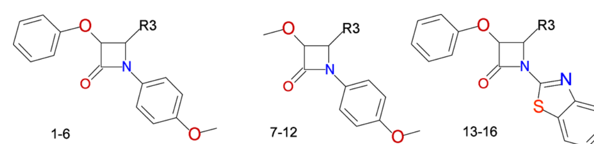


Figure 1. General structure of azetidin-2-one derivatives.

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Table 1. Half Maximal Inhibitory Concentration (IC_{50}) Values of the 16 Azetidin-2-one Derivatives on CHANG, SiHa, and B16F10 Cell Lines

compd	R3	SiHa (IC_{50}[a] μM)	B16F10 (IC_{50}[a] μM)	CHANG (IC_{50}[a] μM)
1	*p*-$CH_3OC_6H_4$–	0.42 ± 0.03	9.7 ± 0.12	0.63 ± 0.03
2	C_6H_5–	0.20 ± 0.04	0.28 ± 0.04	2.85 ± 0.52
3	*p*-$NO_2C_6H_5$–	3.64 ± 0.26	0.87 ± 0.13	2.08 ± 0.41
4	*o*-$NO_2C_6H_5$–	0.22 ± 0.05	4.82 ± 0.33	3.73 ± 0.49
5	*p*-FC_6H_5–	0.08 ± 0.02	3.26 ± 0.18	0.74 ± 0.12
6	*p*-$CH_3C_6H_5$–	**0.07 ± 0.03**	**1.21 ± 0.08**	**>10.0**
7	*p*-FC_6H_5–	2.89 ± 0.37	2.82 ± 0.43	7.57 ± 0.61
8	*p*-$NO_2C_6H_5$–	8.13 ± 0.28	2.08 ± 0.48	>10.0
9	*o*-$NO_2C_6H_5$–	7.29 ± 0.31	2.91 ± 0.15	9.75 ± 0.56
10	*p*-$CH_3OC_6H_4$-	6.36 ± 0.37	2.05 ± 0.45	8.38 ± 0.69
11	*p*-ClC_6H_4–	8.61 ± 0.71	>10.0	3.41 ± 0.34
12	*p*-$CH_3C_6H_4$–	0.73 ± 0.08	4.12 ± 0.16	3.92 ± 0.37
13	*p*-$NO_2C_6H_5$–	0.12 ± 0.04	0.45 ± 0.09	0.07 ± 0.03
14	C_4H_3O	0.33 ± 0.02	0.34 ± 0.03	0.017 ± 0.01
15	*p*-ClC_6H_4–	0.09 ± 0.05	0.31 ± 0.07	0.23 ± 0.06
16	C_5H_4N	1.34 ± 0.11	1.70 ± 0.13	1.03 ± 0.43
vincristine		0.01 ± 0.005	0.01 ± 0.002	0.01 ± 0.004

[a]IC_{50} are mean of two or three experiments, and standard deviation is given.

names and characterization results of 16 synthesized compounds are enclosed with this study as a supplementary file.

NMR study of azetidin-2-one derivatives (**1–6**) indicates a cis configuration with two doublet signals in the field from 5.6 to 6.2 ppm with a coupling constant (J) value from 4.4 to 5.1 Hz. Also, compounds **7–12** reflected two doublet signals (4.7 and 5.9 ppm) with a J value of 4.6–5.1 Hz. However, azetidin-2-one derivatives (**13–16**) showed two singlet signals at 4.6 and 4.9 ppm.[17] Therefore, more structural studies need to be conducted in these compounds to know the exact configuration. However, the diastereoselectivity synthesis of β-lactams by Staudinger reaction has been reported to be affected by parameters as conditions of reaction and size of imine C-substitutes,[18] among others. In this study, it was suggested that substituent imine affects the configuration of compounds **13–16**.

Table 1 shows the IC_{50} values of azetidin-2-one derivatives on SiHa, B16F10, and Chang cell lines together with the vincristine values. For example, compounds **2** and **6** showed high activity against SiHa and B16F10 cell lines. This was when a deeper investigation on compound **6** was proposed.

The reasons for choosing and using these cell types are as follows: In the case of SiHa cells, they represent one of the main cancers affecting women worldwide: cervical cancer. B16F10 cells correspond to a line of murine melanoma capable of developing tumors *in vivo* models opening the possibility of extrapolating assays to search the therapeutic effect and other preclinical characteristics of the molecules in the future. Finally, one does observe that Chang cells are a line of hepatocytes widely used as a reference standard since liver is the main organ related to body detoxification.[19]

In terms of the cytotoxic effectiveness, *N*-(1,3-benzothiazol)-3-phenoxy-azetidin-2-one derivatives had the highest activity even when compared to vincristine. However, some compounds obtained from *cis*-*N*-(4-methoxy-phenyl)-3-phenoxy-azetidin-2-one derivatives also showed valuable cytotoxicity in addition to an interesting selectivity to cancer cells as can be seen in Table 1. Here, one can see that the *N*-(1,3-benzothiazol)-3-phenoxy-azetidin-2-one are toxic even for hepatocytes (Chang cells), while some *cis*-*N*-(4-methoxy-phenyl)-3-phenoxy-azetidin-2-one derivatives, particularly compounds **2** and **6**, exhibit selectivity. This specificity is evident when assessing the data of the IC_{50} on cancer cells (SiHa, B16F10) and Chang cell lines.[20]

Comparing the analogue compounds **6** and **12**, the first one of the *cis*-*N*-(4-methoxy-phenyl)-3-phenoxy-azetidin-2-one group and the second one of the *cis*-*N*-(4-methoxy-phenyl)-3-methoxy-azetidin-2-one group, the only difference one finds is in the exchange of phenoxy to methoxy group in the C3 on the ring, and it can be suggested that a phenoxy substitute, a crucial high hydrophobic group, plays an important role in the biological activity, which is apparently lost in the respective small hydrophobic methoxy (see Table 1). The same applies to the compounds **5** and **7**, which have analogue structures in C3 and exhibit a marked difference in cytotoxic activity as it is increased considerably in the presence of phenoxy when compared with methoxy. Under this sole difference, the biological activity is dramatically modified (see Table 1). As mentioned by Frezza et al., substitutions at N1 and C3 of the ring of azetidin-2-one can potentiate the antineoplastic activity.[21]

Compound *cis*-*N*-(4-methoxy-phenyl)-3-phenoxy-4-(4-methyl-phenyl)-azetidin-2-one (**6**) having an IC_{50} of 0.1 μM on SiHa (cervical cancer) and 1.2 μM in B16F10 (melanoma) cells marked, nevertheless, an increased IC_{50} value of 10.00 μM in Chang (hepatocytes) cells. With these data, compound **6** can be suggested as a promising target for subsequent studies, due to its selective anticancer activity and a lower toxicity on liver cells.

Apoptotic activity assay was performed on B16F10 cells treated with compounds **6**, **8**, and **14** as representatives of each group of azetidin-2-one derivatives.[22]

Caspase 3 is a protein that plays a central role into the execution-phase of cell apoptosis. Cleavage site of this enzyme comprises the amino acid sequence Asp-Glu-Val-Asp (DEVD). When the enzyme is active (a conditional event for apoptosis induction) a cascade of programmed cell death reactions is triggered by the cleavage of this amino acid sequence in the substrates. In the spectrophotometric caspase 3 assay, a synthetic Ac-DEVD-pNA reagent is used as the substrate,

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releasing p-nitroanilide as a product whose concentration can be spectrophotometrically measured (λ= 400 nm). At this juncture, the caspase-3 enzymatic activity was calculated to determine the percentage of apoptosis using podophyllotoxin (a well-known apoptotic inductor) as positive control. Results of apoptotic activity are shown in Table 2, and furthermore,

Table 2. Apoptotic Activity of Lead Azetidin-2-one Derivatives versus Podophyllotoxin

compds	apoptotic activity
podophyllotoxin	100% positive
6	100% positive
8	88% positive
14	93% positive
negative (untreated cells)	0% negative

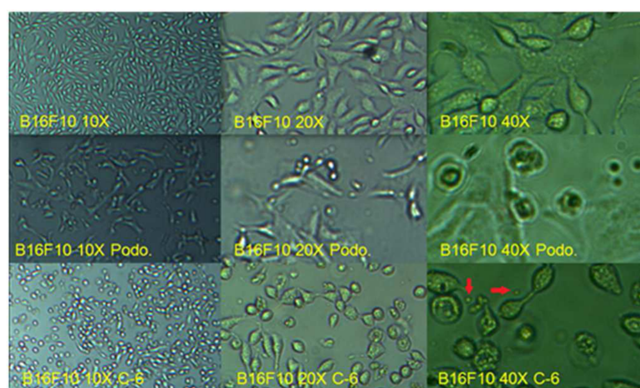

Figure 2. B16F10 cells developing apoptosis with podophyllotoxin or compound **6**.

Figure 2 brings forward a graphic representation of the apoptotic effect on a microscopic slide. The presence of cell death by apoptosis in both cases is clearly shown as the formation of organized shedding vesicles (arrow) after they have experimented fragmentation of genetic material.

According to the data obtained from Banik and collaborators on the study of the mechanism of action of the azetidin-2-ones,[23,24] it was proceeded to assess the apoptotic activity of the compounds, establishing that the compounds derived from azetidin-2-one induce apoptosis in the cell line B16F10. Subsequently, these data would be confirmed with the microarray assay.

GenArise analysis results stated the overexpressed and repressed genes by compound **6** on mouse melanoma B16F10 cells.[25,26] In the range of 1.5 standard deviations of z-score, 676 genes were found repressed and 1524 genes were overexpressed, as shown in the file with the accession number E-MTAB-4351 in the ArrayExpress electronic platform (www.ebi.ac.uk/arrayexpress). Some of these gene-groups encode proteins related to biological pathways with tubulin as a relevant component like cytoskeleton synthesis and remodeling and division of the cell.

In order to trace the biological impact of altered genes, an enrichment analysis was performed by using online program Database for the Annotation, Visualization, and Integrated Discovery (DAVID),[27] and the results (see Supporting Information) brought forward groups of overexpressed genes related to pathways involved in programmed cell death route and synthesis of the cytoskeleton. This suggested that compound **6** interacts with proteins related to these processes, forcing the system to overexpress this kind of genes. Results also showed the down-regulation of genes related to cell division processes meaning that compound **6** could disturb the growth of the cell as a mechanism of cell destruction. It is important to highlight that cytoskeleton synthesis, a process related to the cell division, requires the massive production of tubulin to build the necessary conditions for the new formation of cells. Although a more detailed study of the results is still necessary, it can be strongly suggested that azetidin-2-one derivatives induce apoptosis and damage to the cytoskeleton whose effect is directly related to the interaction between the molecule and the target tubulin as the main component of the microtubules, the base of this cell organelle.

Most studies in the emerging area of cancer therapies with high throughput technologies for gene expression analysis by DNA microarrays are aimed at evaluating the drug-resistance in different cancer cell lines. Hence, results obtained here are quite striking as this work is one of the firsts to seek and elucidate the mechanism of action of a new promising anticancer molecule using DNA microarray analysis. In previous studies, as would be expected, MDR gene overexpression was found, in cases of resistance to paclitaxel and vincristine, as well as of detoxifying enzyme genes. Interestingly, apoptosis inhibiting and cell cycle inducing genes were also found in contrast to results shown here, but in total agreement with the model since in such studies an effect of drug resistance is induced in the system, while in the present work, a well-documented pro-apoptotic and cytotoxic effect is confirmed.[28,29]

Microtubules are the main constituent of the mitotic spindle and are composed of the α/β heterodimeric protein tubulin. Microtubules are a highly validated target in cancer therapy, and a large number of chemically diverse substances alter microtubule polymerization and dynamics by binding to tubulin protein. Tubulin has three well-characterized binding sites: the taxane domain (in alpha-tubulin), the vinca domain, and the colchicine domain (both in beta tubulin). Colchicine binding site has been previously suggested as the site where compounds derived from azetidin-2-one interact with tubulin.[30]

In order to explore the interactions among tubulin and our synthesized derivatives, we performed docking simulations. The pig tubulin (PDB ID: 5CB4) was selected as the receptor for docking studies because it shows 100% of sequence identity with human tubulin and possess high resolution.

The colchicine binding site is surrounded by two α-helices (H7 and H8) and by strands of the two tubulin β-sheets (S1–S4–S5–S6 and S7–S10–S8–S9) from the β subunit capped by two loops.[31] This binding site was selected, and molecular docking simulations of azetidin-2-one derivatives were performed on it. The colchicine and vincristine molecules were used as a positive and negative control, respectively. A pattern of coupling free energy kcal/mol similar to colchicine was found (Table 3).

Several studies have revealed the significance of these residues outlining the binding site for positioning of the compounds and their biological activities.[32−36]

The analysis of the docking results showed that the derivatives used in this study interact frequently with the residues mentioned above, and with others that are highly conserved in the sequences of the tubulins. This was the case for VAL236, CYS239, ASP249, ASN256, ARG58, GLY80,

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Table 3. Molecular Docking Energy of the Compounds Derived from Azetidin-2-one in the Binding Site of the β-Tubulin

compds	Vina score[31] (kcal/mol)	compds	Vina score[31] (kcal/mol)
1	−8.4	**10**	−8.2
2	−8.9	**11**	−8.2
3	−8.5	**12**	−8.4
4	−9.1	**13**	−9.1
5	−9.4	**14**	−8.2
6	−8.5	**15**	−9.1
7	−8.5	**16**	−8.1
8	−8.2	**colchicine**[a]	−8.3
9	−8.6	**vincristine**[b]	30.6

[a]Positive control. [b]Negative control.

ARG117, and ASN101, THR179 from beta and alpha subunits, respectively. Most interactions between these residues and the derivatives were driven mainly by hydrophobic forces (Figure 3a). ASN256 and ASN101 could also play a role as strong

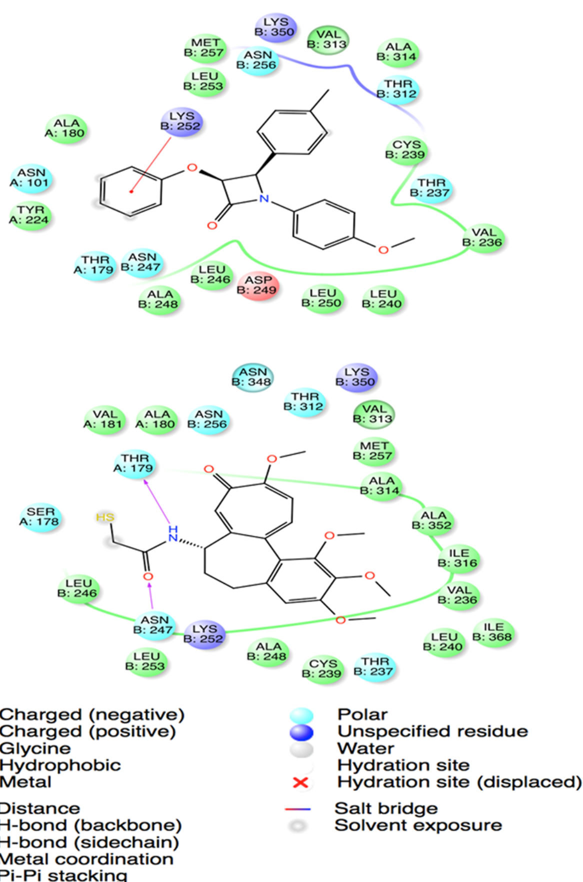

Figure 3. Molecular interactions of (a) compound **6** and (b) colchicine with the human α/β tubulin (PDB ID: 5CB4), respectively.

hydrogen bond donors demonstrated elsewhere.[32−36] The results of the interactions of our derivatives into colchicine binding site were consistent with previous results found for colchicine and other colchicine binding site inhibitors[32−36] (Figure 3a,b). These interactions include a mix of hydrophobic, charged, and polar residues, highlighting the pi-cation interaction with LYS252 and with polar patch formed by the THR179, ASN256, and THR312 residues. Therefore, the results of interaction for the derivatives of this study suggest that they probably share the same inhibition mechanism as that of colchicine and other colchicine binding site inhibitors.

In conclusion, *cis*-*N*-(4-methoxy-phenyl)-3-phenoxy-azetidin-2-one derivatives have anticancer activity on the cell lines SiHa and B16F10 showing selectivity when compared to the cell line Chang in the case of compound **6**. These findings make this molecule a promising compound compared to vincristine, one of the most widely used therapy to treat patients with cancer, according to its IC_{50} value. Apoptotic activity of representative compounds of each group of derivatives of azetidine-2-one, through caspase-3 assay, was demonstrated. It was found that compound **6** induces a gene expression pattern related to pathways associated with cell death due to apoptosis, supporting experimental caspase-3 detection assays. This molecule also induces genes related to remodeling of the cytoskeleton, which is directly related to tubulin production. This suggests that compound **6** interacts with proteins related to this process, forcing the system to overexpress this kind of genes to compensate the deficiency caused by its interaction. Ligands corresponding to studied derivatives of azetidin-2-one could have a potential affinity for the binding site of colchicine to the β-tubulin, involving interactions with key residues outlining this binding site. However, further analysis that includes the demonstration of the effect of the compounds on tubulin polymerization/depolymerization is necessary to deduce this hypothesis further. Compound **6** has a similar predicted affinity that the colchicine exhibits specificity for cancer cell lines.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.6b00313.

Database deposition file with the accession number E-MTAB-4351 in the ArrayExpress electronic platform; ^{1}H NMR and IR spectral data; elemental analysis data; three tables enlisting the group of altered genes by compound **6** in cell line B16F10 involved in cell death pathway; synthesis of the cytoskeleton and cell division processes (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. F.O. carried out the cytotoxic, apoptotic, and microarray experimental assays as well as part of the synthesis experiments, G.R. proposed, supervised, and acquired funding to the synthesis, A.M.P.V. and C.M.M.R. carried out part of the synthesis process, A.S.C. supervised the computational analysis, and I.B.R. supervised the biological experimental work and carried out general supervision of the research group and acquisition of funding. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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