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Biological activity of 3-chloro-azetidin-2-one derivatives having interesting antiproliferative activity on human breast cancer cell lines

Adele Chimento [a,†], Marina Sala [b,†], Isabel M. Gomez-Monterrey [c], Simona Musella [d], Alessia Bertamino [b], Anna Caruso [a], Maria Stefania Sinicropi [a], Rosa Sirianni [a], Francesco Puoci [a], Ortensia Ilaria Parisi [a], Carmela Campana [a], Emilia Martire [a], Ettore Novellino [c], Carmela Saturnino [b,*], Pietro Campiglia [b], Vincenzo Pezzi [a,*]

[a] Department of Pharmacy, Health and Nutrition Sciences, University of Calabria, 87036 Arcavacata di Rende, Cosenza, Italy
[b] Department of Pharmacy, University of Salerno, Via Giovanni Paolo II 132, 84084 Fisciano, Salerno, Italy
[c] Department of Pharmaceutical and Toxicological Chemistry, University of Naples 'Federico II', 8013 Napoli, Italy
[d] Department Pharmaco-Biological, University of Messina, 98168 Messina, Italy

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ABSTRACT

Resveratrol (3,4′,5 tri-hydroxystilbene), a natural plant polyphenol, has gained interest as a non-toxic agent capable of inducing tumor cell death in a variety of cancer types. However, therapeutic application of these beneficial effects remains very limited due to its short biological half-life, labile properties, rapid metabolism and elimination. Different studies were undertaken to obtain synthetic analogs of resveratrol with major bioavailability and anticancer activity. We have synthesized a series 3-chloro-azetidin-2-one derivatives, in which an azetidinone nucleus connects two aromatic rings. Aim of the present study was to investigate the effects of these new 3-chloro-azetidin-2-one resveratrol derivatives on human breast cancer cell lines proliferation. Our results indicate that some azetidin-based resveratrol derivatives may become new potent alternative tools for the treatment of human breast cancer.

Breast cancer is a major cause of death in women, even if disease-free survival and overall survival of patients with breast cancer have been improved through intensive treatment.[1] Major breast cancer treatment methods consist, both separately and in combination with surgery, radiotherapy and chemotherapy. In particular, the anti-estrogen tamoxifen is widely used in the prevention and treatment of estrogen receptor positive breast cancer.[2] Inherent or acquired tumor drug resistance limits many agents that could be used to treat this disease and are often associated with severe, dose-limiting and systemic toxicities. Therefore, new agents acting on novel targets in breast cancer are currently under investigation. Interest in the pharmacological effects of bioactive compounds on cancer treatment and prevention has increased dramatically in recent years. A great number of natural agents derived from plants are studied as agents potentially useful in combined therapy for cancer patients. As well, there is a need to develop new more powerfully active drugs with reduced side effects that can substitute current pharmacological therapies.

Several studies confirmed that increasing vegetable and fruit consumption might reduce the risk of breast cancer.[3] Also, a lower incidence of breast cancer is associated with a high consumption of phytoestrogens,[4] which are biologically active plant-derived phenolic compounds that structurally mimic the mammalian estrogen, 17β-estradiol.[5]

Among many bioactive compounds, basic and preclinical researches on resveratrol, a non-flavonoid polyphenolic compound abundant in grapes, peanuts and other foods that are commonly consumed as part of human diet,[6] have shown a broad range of advantageous biological actions, including cardioprotection[7] and prolongation of lifespan in several species.[8] Resveratrol is a phytoalexin that in nature protects the plant from injury, ultraviolet (UV) irradiation, and fungal attack.[9] Resveratrol exists as cis- and transisomeric forms, with trans to cis isomerization facilitated by UV exposure. Its stilbene structure is related to the synthetic estrogen

* Corresponding authors. Tel.: +39 0984 493148; fax: +39 0984 493271 (V.P.). tel.: +39 089 969769; fax: +39 089 969602 (C.S.).
E-mail addresses: saturnino@unisa.it (C. Saturnino), v.pezzi@unical.it (V. Pezzi).
† These authors contributed equally to this work.

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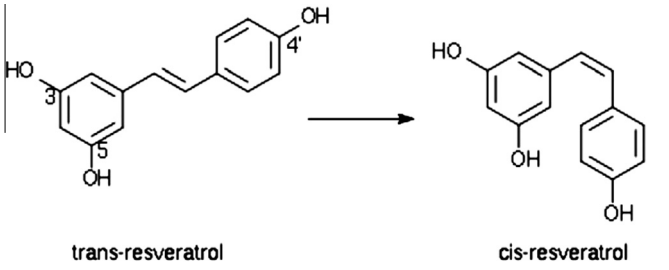


Figure 1. Chemical structure of resveratrol (RSV).

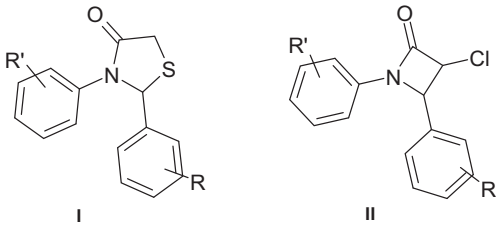


Figure 2. Structures of 2,3-thiazolidin-4-one derivatives (**I**) and of 3-chloro-azetidin-2-one derivatives (**II**).

diethylstilbestrol. Two phenol rings are linked by a styrene double bond to generate 3,4′,5-trihydroxystilbene (Fig. 1).

The biological properties of resveratrol are attributed to its ability to inhibit the oxidation of human low-density lipoprotein, while suppression of cycloxygenase-2 and inducible nitric oxide synthase activities also contribute to its anti-inflammatory and antioxidant effects.[10] Furthermore, the chemopreventive effect of resveratrol is thought to be due to inhibition of quinone reductase 2 activity, which in turn up-regulates the expression of cellular antioxidant and detoxification enzymes to improve cellular resistance to oxidative stress.[11] Resveratrol also increases the activity of SIRT (a member of the sirtuin family of nicotinamide adenine dinucleotide-dependent deacetylases), resulting in improved cellular stress resistance and longevity,[8,10,12] Jang et al.[13] have suggested that it inhibits all three phases of tumor development: initiation, promotion, and progression in various cancers such as prostate,[13] colon,[14] endometrial,[15] hepatocarcinoma[16] and breast cancer.[17,18] Resveratrol contains strong anti-proliferative properties in many cultured cancer cell lines, and acts both by arresting cell cycle and by inducing apoptosis,[19–23] but the apoptosis inducing effects of resveratrol seemed diverse on different tumor cells.[19–21] Resveratrol has been shown to interfere with signal transduction pathways, to modulate cell cycle regulating proteins and to induce apoptosis in multiple cancer cell lines with various mechanisms, including through a p53-dependent pathway and PKC/Akt pathway.[24]

However, therapeutic application of these beneficial effects of resveratrol remains very limited due to its short biological half-life, labile properties, and rapid metabolism and elimination.[10] In fact, in human and rodent, three metabolic pathways have been identified, that is, sulfate and glucuronic acid conjugation of the phenolic groups and hydrogenation of the aliphatic double bond, the latter likely produced by the intestinal microflora.[25] Extremely rapid sulfate conjugation by the intestine/liver appears to be the rate-limiting step in resveratrol's bioavailability.[26] Analysis of recent literature reveals an increasing number of formulations under study, which reflects the major interest in developing pharmaceutical forms able to improve resveratrol bioavailability as a step towards applying its therapeutic potential in vivo.[26]

Several studies were undertaken to obtain resveratrol synthetic derivatives with potent anticancer activity, enhanced structural rigidity and major bioavailability.[27] Structure-activity studies have revealed crucial elements of the parental components that are required for specific effects. To give one example, the 4-hydroxy group in the trans conformation on the 4- and 4′-positions of the stilbenic backbone and the methoxy groups added to the trihydroxystilbene scaffold of resveratrol have been identified as crucial for antiproliferative[28] or cytotoxic[29] resveratrol effects, respectively. In addition, the structural replacement of the stilbene moiety of resveratrol has been found to be a promising strategy for thegeneration of synthetic analogues with improved pharmacokinetic parameters. Mayhoub et al.[30] described a series of trans and cis 2,3-thiadiazol analogs of resveratrol using as design strategy the replacement of the alkene linker between the two aromatic rings with a heterocyclic system. Starting from this approach, recently we have synthesized 2,3-thiazolidin-4-one resveratrol analogues that have increased structural rigidity and potent activity (I Fig. 2) fixing the cis-conformation of resveratrol, hypothesizing that also using cis stilbene template could have active derivatives. We demonstrated that some derivatives displayed stronger antiproliferative effects than resveratrol in human breast cancer cells.[27]

Table 1
Bioaccessibility (%) of **4a–f** compounds

Sample	Bioaccessibility (%)
Resveratrol	31 ± 0.9
4a	79 ± 0.8
4b	78 ± 0.9
4c	83 ± 1.1
4d	87 ± 0.7
4e	80 ± 1.0
4f	70 ± 0.9

In the present work, we prepared a new series of 1,4-diaryl-3-chloro-azetidin-2-one derivatives in which a azetidin-2-one nucleus connects two aromatic rings (II Fig. 2); also these new compounds have structural rigidity, major bioavailability and antiproliferative activity.

The designed compounds (**4a**–f, Table 1)[31] were prepared by the following Staudinger reaction.[32] Cycloaddition of imines (**3**) with 2-chloro-acetylchloride in the presence of triethylamine afforded beta-lactams **4a–f** in high yields (47–93%). The imines were obtained by condensation of aldehydes (**1**) and amines (**2**) in toluene (Scheme 1).

The bioavailability of the new synthesized 3-chloro-azetidin-2-one resveratrol derivatives was measured by dialysis tubing procedure,[33,34] a quick and low cost method that represents a good model to evaluate the bioavailability of different kinds of molecules.

In most cases, the oral route represents the most convenient one for drug administration and, thus, it is important to choose an appropriate model to investigate absorption and bioavailability of therapeutic agents in the gastrointestinal system.

Bioavailability was defined as the percentage of tested compounds recovered in the bioavailable fraction, after in vitro digestion, in relation to the original non-digested samples.

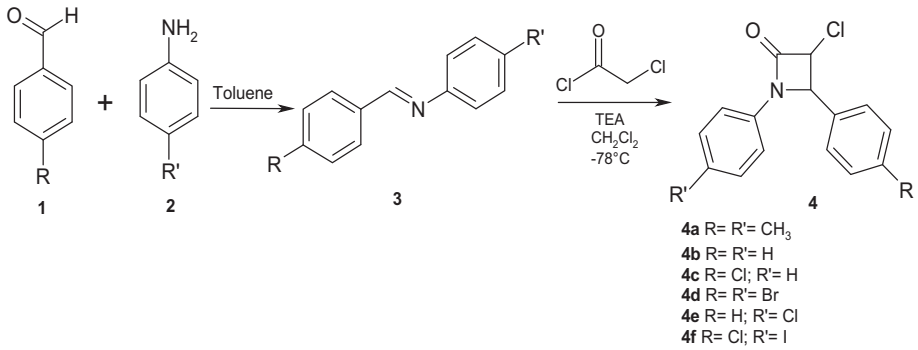
This value can be calculated by the following equation (1):

(bioavailable content/total content) × 100 (**1**).

The obtained results, after six hours, were reported in Table 1.

As it is possible to note, all the synthesized compounds showed a higher bioavailability compared to resveratrol, which was more than 2 times less bioavailable, and the best results were observed for **4d** with about 87% of the initial content recovered in the bioavailable fraction.

The higher bioavailability of these novel 3-chloro-azetidin-2-one resveratrol derivatives could be due to their modified struc-



Scheme 1. Synthesis of 3-chloro-azetidin-2-one derivatives (**4**).

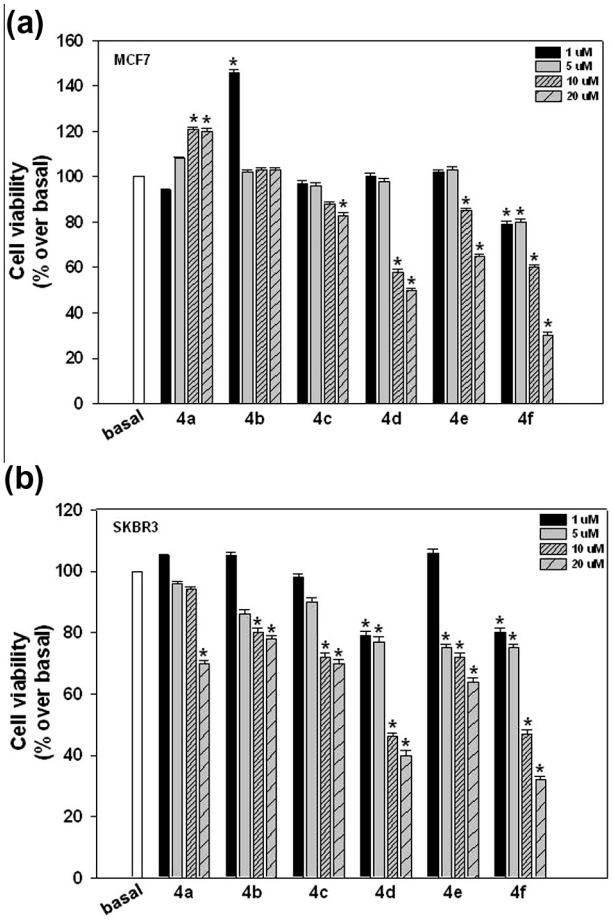


Figure 3. Effects of different doses of 3-chloro-azetidin-2-one derivatives on MCF-7 (a) and SKBR3 (b) cell proliferation. Cells were treated for 72 h with the indicated concentrations of compounds **4a–f**. Cell viability was evaluated by MTT assay. Statistically significant differences are indicated. Columns, mean of three independent experiments each performed with triplicate samples expressed as percent of basal, bars, SD; (*P <0.01 compared with basal).

tures making these molecules more lipophilic compared to resveratrol.

Starting from bioavailability results of **4a–f** compounds, we evaluated its effects on cell proliferation against estrogen receptor positive (ER+) MCF-7 and estrogen receptor negative (ER−) SKBR3 human breast cancer cell line (Fig. 3) at different concentration (1, 5, 10, 20 μM) using the MTT assay.[35] Therefore, we treated for 72 h MCF-7 and SKBR3 cells with each compounds and also with RSV in order to compare the antiproliferative effects of the chemicals used to this well-known anticancer agent.[35–41]

Table 2
IC_{50} of resveratrol and its derivatives **4d** and **4f** for MCF7 and SKBR3 cells on cell viability

Compounds	MCF7		SKBR3	
	IC_{50} (μM)	95% Confidence interval	IC_{50} (μM)	95% Confidence interval
4d	16.72	13.42–20.84	11.09	8.37–14.68
4f	11.77	8.93–15.52	9.51	7.69–11.75
RSV	28.38	25.71–31.33	41.22	34.21–49.67

As showed in Figure 3, in MCF7 cells (Fig. 3a) **4d–f** compounds, determined a clear dose-dependent inhibitory effect on cell growth. Higher doses of **4a** compound elicited an stimulation while only low dose of **4b** compound favored it. However, **4c** compound decreased cell viability only at 20 μM concentration.

Among all tested compounds in SKBR3 cells (Fig. 3b), higher concentrations of **4b**, **4c**, **4e** derivatives elicited a negative inhibitory effect. Remarkably, a 72 h treatment with **4a** compound (20 μM) was able to prevent SkBr3 cell growth. It should be pointed out that **4d** and **4f** compounds inhibited the proliferation in a dose dependent manner in both estrogen dependent MCF-7 and in estrogen independent SKBR3 cell lines suggesting that these compounds could be potentially active in different breast cancer subtypes.

As showed in Figure 3, **4d**, **4f** compounds are more active, as also evidenced by its half-maximal inhibitory concentration (IC50) values (Table 2) that are much more relevant respect to IC50 value of RSV.

In fact, it is evident that the dose at which RSV shows antiproliferative effects on MCF7 and SKBR3 cells (Supplementary data Fig. S1), is relatively higher (>20 μM) (Table 2)[42] compared to that of the tested compounds.

On the basis of the aforementioned promising results obtained in two different model systems of breast cancer cells, the capability of **4d** and **4f** compounds to elicit strong repressive effects on breast cancer cell growth, could be determined by presence, as electron-attractor atoms, of two halogens on aromatic rings.

In addition, a control experiment using 3T3 mouse embryonic fibroblast cells has been performed; no effects on cell viability was obtained using all azetidin-based resveratrol derivatives of 5 μM from to 20 μM after 72 h of treatment (Supplementary data Fig. S2), suggesting that these compounds have specific inhibitory effect on breast cancer cells.

In conclusion, considering the widespread chemopreventive and chemotherapeutic applications of resveratrol, a strong demand exists to search for other pharmacologically active resveratrol analogs with enhanced bioavailability, potency and selectivity. For such reason, a series of new 3-chloro-azetidin-2-one derivatives

has been synthesized and evaluated for their growth regulatory effects in MCF7 and SkBr3 human breast cancer cells. Among these compounds, that showed moderate to high antitumor activity and displayed a more in vitro bioavailability respect to resveratrol, the strongest antiproliferative activity against human breast cancer cells tested was displayed especially by **4d** and **4f**. Hence, the capability of these compounds to have greater bioavailability than resveratrol and to elicit selective inhibitory effects on breast cancer cell growth could be taken into account towards novel pharmacological approaches in breast cancer therapy. Experiments useful to investigate the molecular mechanism involved and to evaluate in vivo bioavailability and chemotherapeutic potential of compounds are in progress.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.09.054.

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31. *Experimental section:* Reagents, starting material and solvents were purchased from Sigma–Aldrich (Milano, Italy) and used as received. Analytical TLC was performed on plates coated with a 0.25 mm layer of silica gel 60 F254 Merck and preparative TLC on 20 × 20 cm glass plates coated with a 2 mm layer of silica gel PF254 Merck. Silica gel 60 (300–400 mesh, Merck) was used for flash chromatography. Melting points were measured with a Köfler apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded with a Bruker 300 spectrometer operating at 300 and 75 MHz, respectively. Chemical shifts are reported in δ values (ppm) relative to internal Me4Si and J values are reported in Hz. Mass spectra were obtained using a ESI mass spectrometer: Finnigan LCQ Advantage max (Thermo Finnigan; San Jose, CA, USA).
*General procedure for the synthesis of imine (**3**).* To a solution of the arylamine (10 mmol) in toluene (40 mL) was added aryl-aldehyde (12 mmol) and the mixture was refluxed overnight using a Dean–Stark water separator (monitored by TLC). When the reaction was over, toluene was evaporated under reduced pressure, and the crude product was used as such for the next reaction.
*General Procedure for the synthesis of β-lactam (**4**).* A solution consisting of 2-chloro-acetylchloride (1.5 mmol) in dichloromethane (10 mL) was added drop wise to a stirred solution containing imine (1 mmol) and distilled triethylamine (3 mmol) in dry dichloromethane (10 mL) at −78 °C. The reaction mixture was then stirred overnight at room temperature, washed with saturated sodium bicarbonate solution (10 mL), dilute hydrochloric acid (10%, 10 mL), brine (10 mL), dried with anhydrous sodium sulfate, and evaporated to obtain the crude product. The pure product (48–68%) was then isolated via column chromatography over silica gel using ethyl acetate:hexanes (1:4) as the solvent.
*3-Chloro-1,4-di-p-tolylazetidin-2-one (**4a**):* Yellow solid. Yield 51%. Mp 103–104 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.29 (d, 6H), 5.27 (d, J = 6 Hz, 1H), 5.39 (d, J = 6 Hz, 1H), 7.07 (m, 2H), 7.22–7.26 (m, 4H), 7.28 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 21.3, 62.0, 68.1, 125.3, 129.2, 133.4, 136.4, 136.5, 136.8, 140.5, 162.2. ESI m/z calcd for 285.77; found: 286.04. Anal. Calcd for $C_{17}H_{16}ClNO$: C, 71.45; H, 5.64. Found: C, 71.48; H, 5.62.
*3-Chloro-1,4-diphenylazetidin-2-one (**4b**).* White solid. Yield 61%. Mp 189–190 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.30 (d, J = 3 Hz,1H), 5.43 (d, J = 6 Hz, 1H), 7.13 (m, 2H), 7.26–7.40 (m, 6H), 7.43 (m, 2H).^{13}C NMR (75 MHz, $CDCl_3$): 62.0, 68.1,117.7, 126.7, 126.9, 128.0, 128.5, 129.9, 139.5, 143.5,162.2. ESI m/z calcd for $C_{15}H_{12}ClNO$: 257.71; found: 257.95. Anal. Calcd for $C_{15}H_{12}ClNO$: C, 69.91; H, 4.69. Found: C, 69.94; H, 4.71.
*3-Chloro-4-(4-chlorophenyl)-1-phenylazetidin-2-one (**4c**).* White solid. Yield 48%. Mp 178–179 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.33 (d, J = 12 Hz, 1H), 5.41 (d, J = 6 Hz, 1H), 7.14 (m, 2H), 7.26–7.31 (m, 5H), 7.40 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): 62.0, 68.1, 117.7, 127.2, 128.6, 128.0, 128.9, 132.3, 139.5, 141.6, 162.2. ESI m/z calcd for $C_{15}H_{11}Cl_2NO$: 292.16; found: 292.08. Anal. Calcd for $C_{15}H_{11}Cl_2NO$: C, 61.67; H, 3.79. Found: C, 61.70; H, 3.81.
*1,4-Bis(4-bromophenyl)-3-chloroazetidin-2-one (**4d**).* White solid. Yield 59%. Mp 163–164 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.30 (d, J = 3 Hz, 1H), 5.39 (d, J = 6 Hz, 1H), 7.17–7.20 (m, 4H), 7.44 (m, 2H), 7.56 (m, 2H).^{13}C NMR (75 MHz, $CDCl_3$):62.0, 68.1, 121.1, 122.3, 127.2, 131.4, 131.8, 136.7, 138.5, 142.5, 162.2. ESI m/z calcd for $C_{15}H_{10}Br_2ClNO$: 415.51; found: 415.03. Anal. Calcd for $C_{15}H_{10}Br_2ClNO$: C, 43.36; H, 2.43. Found: C, 43.34; H, 2.40.
*3-Chloro-1-(4-chlorophenyl)-4-phenylazetidin-2-one (**4e**).* White solid. Yield 65%. Mp 156–157 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.30 (d, J = 3 Hz, 1H), 5.41(d, J = 6 Hz, 1H), 7.31–7.44 (m, 7H), 7.54 (m, 2H).^{13}C NMR (75 MHz, $CDCl_3$): 62.0, 68.1, 86.1, 123.2, 127.2, 128.6, 132.3, 137.8, 138.4, 141.6, 162.2. ESI m/z calcd for $C_{15}H_{11}Cl_2NO$: 292.17; found: 292.04. Anal. Calcd

for $C_{15}H_{11}Cl_2NO$: C, 61.67; H, 3.79. Found: C, 61.70; H, 3.77.

3-Chloro-4-(4-chlorophenyl)-1-(4-iodophenyl)azetidin-2-one (**4f**). Yellow solid. Yield 68%. Mp 191–193 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.30 (d, J = 6 Hz, 1H), 5.40 (d, J = 6 Hz,1H), 7.06 (m, 2H), 7.25 (m, 2H), 7.40 (m, 2H), 7.60 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$):62.0, 68.1, 86.1, 123.2, 127.2, 128.6, 132.3, 137.8, 138.4, 141.6, 162.2. ESI m/z calcd for $C_{15}H_{10}Cl_2INO$: 418.06; found: 418.10. Anal. Calcd for $C_{15}H_{10}Cl_2INO$: C, 43.10; H, 2.41. Found: C, 43.08; H, 2.43.

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33. *In vitro bioavailability studies:* In vitro bioavailability studies in simulated gastric and intestinal fluids were carried out by performing a slight modified version of the dialysis tubing procedure.[34]

The dialysis tubing method is characterized by two consecutive enzymatic digestions: pepsin and pancreatin digestion, respectively. These steps are described as follows.

Pepsin Digestion. 100 μL of each sample (**4a**–**f**, 10 mM in DMSO) were mixed with 1.0 mL of a 0.85 N HCl solution containing 24,000 U of porcine pepsin per mL and 3 mL of a sodium cholate solution (2% w/v in distilled water). The obtained mixture was introduced into a dialysis bag (Spectrum Laboratories Inc., MWCO: 12–14,000 Dalton, USA) which was then carefully closed and immersed inside a flask containing 10 mL of a 0.85 N HCl solution (pH 1.0). The flask was then incubated into a shaking water bath at 37 °C to simulate the human body conditions of temperature for 2 h.

Pancreatin Digestion. At the end of the 2 h pepsin digestion, the dialysis bag was opened and 11 mg of amylase, 11 mg of esterase and 1.3 mL of a 0.8 M $NaHCO_3$ solution containing 22.60 mg porcine pancreatin/mL were added to the peptic digesta. After the digesta and enzyme solution were well-mixed, the dialysis bag was sealed on each end with clamps and placed into a flask with 10 mL of buffer solution at pH 7.0. The flask was incubated into the shaking water bath at 37 °C for further 4 h.

In the aim to evaluate the bioavailability of the different samples, 2 mL of the medium were withdrawn from the flask for sample analysis at the time points of two and six hours, after pepsin and pancreatin digestions respectively. The concentration of the samples was determined by UV/VIS spectroscopy (UV/VIS spectrometer V-530, Jasco—U.S.) and the percentages were calculated by using the equations obtained from the calibration curves of five standard solutions, for each tested compound, at pH 1.0 and 7.0, respectively.

For this purpose, all the prepared standard solutions were analysed by UV–Vis spectrophotometer and the correlation coefficient (R2), slope and intercept of the regression equations obtained by the method of least square were calculated at pH 1.0 and 7.0, respectively. Each experiment was performed in triplicate.

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35. *Cell culture and treatments.* MCF-7 breast cancer cells (a ER positive breast cancer cells, obtained from American Type Culture Collection (ATCC), Manassas, VA, USA) were maintained as previously described.[36] SKBR3 breast cancer cells (a ER negative breast cancer cells, obtained from American Type Culture Collection (ATCC), Manassas, VA, USA) were maintained in RPMI1640 without phenol red supplemented with 10% fetal bovine serum (FBS), 1% glutamine and 1% penicillin/streptomycin (Sigma–Aldrich, Milano, Italy) (complete medium).[37] 3T3 mouse embryonic fibroblast cells, obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), were maintained in DMEM with phenol red supplemented with 10% fetal bovine serum (FBS), 1% glutamine and 1% penicillin/streptomycin (Sigma–Aldrich, Milano, Italy) (complete medium). Cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 and were screened periodically for Mycoplasma contamination. All 3-chloro-azetidin-2-one derivatives compounds were dissolved in dimethylsulfoxide (DMSO) (Sigma, St. Louis, Missouri, USA) at a concentration of 10 mM and diluted in DMEM/F12 (for MCF-7 cells), in DMEM (for 3T3 cells) or in RPMI (for SKBR3 cells) medium supplemented with 1% DCC-FBS (dextran-coated charcoal-treated newborn calf serum serum) to obtain the working concentration.

Assessment of cell viability. MCF-7, SKBR3 and 3T3 cells were seeded on twenty-four well plates (0.2×10^5 cells/well) and grown for 48 h in complete medium. Before being treated, cells were starved in DMEM/F12 (for MCF-7 cells), in DMEM (for 3T3 cells) or in RPMI (for SKBR3 cells) serum free medium for 24 h to the purpose of cell cycle synchronization. The effect of the different doses of 3-chloro-azetidin-2-one derivatives was measured using (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT) assay as previously described.[27,36–42] Seventy two hours after treatments, fresh MTT (Sigma), re-suspended in PBS, was added to each well (final concentration (0.33 mg/mL). After 3 h incubation, cells were lysed with 1 mL of DMSO. Each experiment was performed in triplicate and the optical density was measured at 570 nm in a spectrophotometer. Statistical analyses. All experiments were conducted at least three times and the results were from representative experiments. Data were expressed as mean values ± standard deviation (SD), and the statistical significance between control (basal) and treated samples was analyzed using the GraphPad Prism 5 software program. The unpaired Student's t-test was used to compare two groups. P <0.05 was considered statistically significant.

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