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2017, 8, 755Design and synthesis of cenocladamide analogues
and their evaluation against breast cancer cell
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This work describes the total synthesis of the alkaloid cenocladamide and a concise library of nine structural analogues aiming at their evaluation against the breast cancer cell line MDA-MB-231. The most promising compound (**3**; IC₅₀ = 6.6 μM) was also evaluated in a panel of seven breast cancer cell lines and two non-tumorigenic cell lines. We further conducted an initial investigation on the mechanism of action of analogue **3**, which lacks the endocyclic double bond when compared to cenocladamide. The present study presents the discovery of a cenocladamide analogue with interesting cytotoxic activity, which could be useful for further optimization towards new chemotherapeutic agents for breast cancer treatment.

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Introduction

Breast cancer is the second most common cancer worldwide after lung cancer, the fifth most common cause of death related to cancer, and the leading cause of cancer death in women. The global burden of breast cancer exceeds all other types of malignancies and the incidence rates of breast cancer are increasing.¹ Although recent advances have been made in tumor detection and treatment, breast tumors are still very challenging in view of their heterogeneity.^{2,3}

Breast cancer treatment includes several strategies from hormone therapy drugs, to surgery, radio- and chemotherapy.⁴ However, resistance of tumor cells to current drugs available for breast cancer treatment highlights the need for new chemotherapeutic agents.⁵

In this context, compounds of natural origin play a key role in the development of novel therapeutics mainly because of their tremendous structural diversity, serving as unique and privileged scaffolds for drug discovery.⁶ Indeed, natural products have a profound impact in several areas, including cancer, and it is estimated that *ca.* 80% of the antitumor agents introduced in the past decades are of natural origin or

inspired by natural compounds.⁷ When allied with organic synthesis, the potential to obtain more powerful derivatives that are not available from natural sources can be leveraged. Total synthesis still has an import role in the continuing exploitation of natural products as lead compounds for drug discovery, as it often represents the most efficient means, even for complex structures, of providing sufficient quantities of materials for meaningful biological profile assessments.⁸ On the other hand, modern and less costly strategies have been used by organic and medicinal chemists to generate derivatives such as molecular simplification^{9,10} and diverted total synthesis, where selected portions of the prototype molecule are modified to understand their importance for the expression of a certain biological activity of a particular class of natural products.^{11,12}

In this study, we employed both strategies to produce a concise series of analogues of the alkaloid cenocladamide. Since its isolation in 2000 by Dodson and co-workers from the leaves of *Piper cenocladum*,^{13,14} only studies of its effect on certain herbivores have been conducted.^{15–17} Cenocladamide is structurally similar to piplartine (also known as piperlongumine, see Fig. 1), a compound that selectively kills cancer cells while sparing primary normal cells, causing an increase in reactive oxygen species (ROS)

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Fig. 1 Chemical structures of cenocladamide and piplartine.

and decrease in glutathione (GSH).^{18,19} Despite this structural similarity, to the best of our knowledge, no study concerning the evaluation of cenocladamide's potential as a new pharmacological agent has been reported so far.

Herein, we report the total synthesis of cenocladamide and a series of analogues aiming at the evaluation of their effect on several breast tumour cell lines (*e.g.* MDA-MB-231, SKBR3, HS578T, HCC38, MDA-MB-468, BT549, T47D and MCF-7) in comparison with non-tumorigenic breast cell lines (*i.e.* MCF10A and iHMEC). Some structural requirements for the expression of the cytotoxic activity were identified and preliminary studies were performed in an attempt to unveil the mechanism responsible for cell death.

Therefore, the design of cenocladamide analogues was planned to evaluate the importance of the structural features of this natural product related to their biological activity regarding cancer cells (Fig. 2). First, we investigated the effect of replacing a phenolic hydroxyl by a methoxy group (compound 2), to mimic the side chain of piplartine. The influence of methoxy and hydroxyl groups on the cytotoxic activity has been extensively investigated and there are several natural compounds with different degrees of methoxylation and/or hydroxylation with interesting antitumor properties, such as resveratrol, combretastatin, curcumin, etoposide, and others.²⁰ Further, the role of this replacement was also evaluated in compounds 4 and 6, and these groups were removed in compound 9. Additionally, we explored the modification of the nitrogenated heterocycle, removing the endocyclic double bond (compounds 3 and 4), and also removing the double bond and the ketone group (compounds 5 and 6). Next, a molecular simplification strategy was employed to verify the importance of the side chain (compounds 7–8). Thus, the *N*-cinnamoyl group was replaced with simple acetyl and benzoyl groups. Finally, removal of the exocyclic double bond resulted in analogue 10, which could allow the evaluation of the importance of this electrophilic site for selectivity and cytotoxicity. Adams *et al.* synthesized and tested an array of piplartine analogues and identified the *endo* double bond as

a key pharmacophore, with the *exo* double bond also playing a significant role in determining cytotoxicity.²¹

Results and discussion

Chemistry

Firstly, the 2,3-dihydro-4-pyridone core present in compounds 1–2 and 7–10 was constructed based on the protocols reported by Brimiouille and Bach.²² Thus, 4-methoxypyridine (11) was treated with benzyl chloroformate, followed by a reduction of the acyl pyridinium intermediate to obtain *N*-Cbz-2,3-dihydro-4-pyridone 12 in 68% yield. Next, hydrogenolysis under standard conditions removed the nitrogen protecting group to generate 2,3-dihydro-4-pyridone 13 in a quantitative yield (Scheme 1). Then, 2,3-dihydro-4-pyridone 13 was acylated with acetic anhydride or acyl chlorides, which were prepared from the corresponding carboxylic acids upon treatment with oxalyl chloride and DMF, furnishing analogues 2, 7–10 and *O*-acetyl cenocladamide 14. Finally, deprotection of phenolic acetate with ammonium acetate afforded cenocladamide (1),²³ whose spectroscopic data were identical to those reported for the natural sample confirming the proposed structure.¹³

Analogues 5 and 6 were prepared by a coupling reaction between piperidine (15) and the corresponding carboxylic acids, employing carbodiimides as carboxyl activators. A similar procedure was performed using amine 16 to obtain amides 17 and 18, which were then subjected to acidic conditions in order to promote the hydrolysis of the ketal protecting group thus releasing the ketone functionality present in analogues 3 and 4 (Scheme 2).

Study of the effect of cenocladamide analogues on breast cancer cell lines

The development of clinical metastasis remains a significant cause of morbidity and mortality from the disease, and tumor invasion plays a crucial role in this process.²⁴ Therefore, we began our study using the human metastatic breast

Fig. 2 Design of cenocladamide analogues.

Scheme 1 Synthesis of cenocladamide (**1**) and analogues **2**, **7–10**.

cancer cell line MDA-MB-231,²⁵ which is highly invasive in comparison with the frequently studied MCF-7 breast cancer cell line. Cell proliferation/death was evaluated after 3 days of treatment with cenocladamide and its analogues at a concentration of 20 μ M. While most of the compounds did not affect or affected marginally cell proliferation, analogues **9** and **2** inhibited proliferation by $\sim$ 50%. Compound **3**, on the other hand, was the most effective compound, killing around 60% of the cells, indicating that the presence of the non-conjugated ketone and the phenolic group plays a key role in

the cytotoxicity of this family of compounds (Fig. 3A). The IC_{50} of **3** in cell proliferation of MDA-MB-231 was 6.6. μ M (Fig. 3B).

From this preliminary screening, it is clear that the exocyclic double bond is more important for the cytotoxic effect displayed by this family of compounds, since compound **2** inhibited cell growth by $\sim$ 50% while the saturated derivative **10** did not affect cell proliferation. As indicated for other natural products and analogues, the α,β -unsaturated scaffold may act as a Michael acceptor for cysteine residues or other nucleophiles in biological systems.^{19,26} On the other hand, removal of the endocyclic double bond resulted in the most cytotoxic analogue (**3**) of this series, which killed $\sim$ 60% of the cells. This effect becomes more evident when we compare the cytotoxic activity displayed by analogue **3** and the natural compound cenocladamide (**1**). This modification may also change the electronics of the whole molecule since the nitrogen lone pair is no longer involved in cross-conjugation and could affect the strength of the HBA and HBD profile of the carbonyl and the phenolic hydroxyl groups, respectively. The importance of the phenolic hydroxyl group as a HBD is also emphasized if one compares compounds **3** and **4**, since methylation leads to only a marginal effect on cell proliferation induced by compound **4**. Finally, the role of the substituents at the aromatic ring is less evident: while methylation ablates the activity of cenocladamide (**1**), compound **9** lacking any substituent is able to inhibit cell growth by $\sim$ 50% compared to the control. To shed new light on the importance of the structural requirements for the cytotoxicity of this family of compounds, new analogues are still needed.

Considering that different cell lines display different sensitivities toward the same cytotoxic agent, we then determined the overall impact of the treatment with compound **3** over seven other breast cancer cell lines (*e.g.* SKBR3, HS578T, HCC38, MDA-MB-468, BT549, T47D and MCF-7), as well as a non-tumorigenic cell line derived from human fibrocystic

Scheme 2 Synthesis of analogues **3–6**: (a) for R = H, DCC, THF, r.t.; (b) for R = Me, EDCI, Et₃N, DMAP, CH₂Cl₂, r.t.

Fig. 3 Compound series affected growth/survival of breast cancer cell lines. (A) The compound series was tested against the MDA-MB-231 breast cancer cell line at 20 μM for 72 hours. (B) Compound 3 dose response in cell proliferation of MDA-MB-231 cells, after 72 hours of treatment. (C) Eight breast cancer cell lines and 2 non-tumorigenic breast cells were treated with 3 or 5 at 20 μM , or doxorubicin at 1 μM for 72 hours.

mammary tissue (MCF10A)²⁷ and an *in-house* immortalized breast epithelial cell line by expression of hTert (iHMEC). For comparison, we used the chemotherapeutic doxorubicin, considered the most effective agent in the treatment of breast cancer patients.²⁸ Compound 3 induced cell death on all tested cell lines, an effect that was not seen with compound 5, an analogue that lacks the carbonyl group at the heterocyclic ring. Compound 3 killed all cell types with an efficacy varying from 15% to more than 80% of death, while doxorubicin inhibited proliferation from 0.2% to around 50% amongst the tested cell lines (with the exception of iHMEC that presented 23.9% of cell death) (Fig. 3C). The observed effect of doxorubicin on the cell lines is in agreement with the already documented cytostatic action of this drug.²⁹ Although compound 3 also caused cell death to the non-tumorigenic MCF10A and iHMEC cells, they were amongst the least affected cells, pointing out a slight selectivity for tumor cell lines.

Intrigued by the extreme effect of compound 3 on cell viability, we looked at two morphological features that could offer clues on how this analogue affects cells. MDA-MB-231 cell nuclei and mitochondria were stained with the fluorophores

Hoechst and MitoTracker Deep Red, respectively. Compound 3, but not compound 5, increased nuclear fragmentation and mitochondria staining, which indicates potential increased mitochondrial biogenesis (Fig. 4A). The effects on nuclei morphology may indicate pyknosis followed by karyorrhexis, the irreversible condensation of chromatin and nuclear fragmentation, respectively, happening in cells undergoing necrosis or apoptosis,³⁰ whereas an increase in MitoTracker intensity may be related to a cytotoxic process involving increased mitochondrial ROS levels.³¹ To confirm these findings, we investigated cell death by flow cytometry using ethidium bromide (EB, which stains necrotic cells) and acridine orange (AO, a vital dye).³² Both tacrine and compound 3 increased the cell population stained with EB and formed a second AO population less stained than the major population found on control cells (Fig. 4B). Altogether, these data show that 3 imparted severe cell injury that led to cell death.

Convinced that compound 3 was causing cell death, we wanted to verify if it was also stopping cell proliferation. Our cell high-content analysis showed that, even at the highest tested concentration (25 μM), compound 3 did not block cells entering the S (synthesis) or leaving the M (mitosis) phases

Fig. 4 Analogue **3** affected cell nuclear morphology, mitochondrial staining with MitoTracker and led to increased cell death. (A) High-content analysis of MDA-MB-231 treated with compound **3**, but not compound **5**, pointed to increased nuclear fragmentation and, potentially, increased mitochondrial mass. (B) Flow cytometry of MDA-MB-231 cells treated with compound **3** or tacrine showed increased staining for ethidium bromide (a dead cell marker) and decreased staining for acridine orange (a viable cell probe), compared to DMSO incubated cells. P1 is the population selected for analysis, and gates 1 and 2 define AO and EB positive cells, respectively.

(Fig. 5A). These data showed that compound **3** does not affect the cell cycle.

Finally, since compound **3** shares a structural similarity with piplartine, a molecule with the potential of increasing cellular ROS production,¹⁸ and the mitochondria staining assay suggested an increase in mitochondrial mass, we tested whether intracellular ROS were being produced in MDA-MB-

231 cells upon treatment with compound **3**. We verified that compound **3**, but not compound **5**, increased cell ROS generation by about 20% after 3 hours of treatment, an effect that was counteracted by the ROS scavenger *N*-acetyl-L-cysteine (NAC). Curiously, the effect of compound **3** was much less pronounced after 24 hours of treatment (Fig. 5B). These results indicate that treatment with compound **3** leads to a

Fig. 5 Compound **3** did not affect the cell cycle and slightly and transiently raised ROS. (A) Fluorescence microscopy of MDA-MB-231 treated with compound **3** at increasing doses showed no alteration in EdU incorporation (S phase marker) and pHH3 detection (M phase-specific marker). (B) Compound **3**, but not compound **5**, increased by around 20% the intracellular ROS levels only after 3 hours of treatment, an effect that was counteracted by NAC.

modest ROS increase in cells, which is not persistent and may not account for the toxicity displayed by this compound in the panel of breast tumor cell lines used in our study.

Conclusions

A concise library of compounds, including the alkaloid cenocladamide and nine structural analogues, was synthesized and evaluated against the MDA-MB-231 cancer cell line. The chemical modifications performed on the structure of cenocladamide allowed the identification of a more potent analogue (**3**), providing insights into the structure–activity relationship (SAR) for the series of compounds prepared in this study. Indeed, two main features seem to be important for the cytotoxic effect displayed by these compounds: the non-conjugated ketone at the heterocyclic ring in combination

with the exocyclic double bond present on the 3,5-dimethoxy-4-hydroxycinnamoyl (sinapic) side chain. Compound **3** was prepared in just two steps in 46% overall yield and exhibited an IC_{50} equal to 6.6 μM over the proliferation of MDA-MB-231 cells. This analogue also showed cytotoxicity against other seven breast cancer cell lines, and less pronounced cytotoxicity on two non-tumorigenic cell lines. Our initial mechanistic studies indicate that both apoptosis and necrosis are likely taking place during cell death after treatment with compound **3**, and this compound does not affect the cell cycle. ROS generation by compound **3** was evaluated, but only a modest increase was observed and may not explain the toxicity mechanism of this compound. The cytotoxicity of compound **3** toward a wide range of breast cancer cell lines deserves further attention to validate this compound as a potential lead compound for the development of new

candidates for breast cancer treatment. Our work also highlights the usefulness of natural products as a source for the discovery of novel chemical entities with enhanced cytotoxicity for drug development.

Experimental section

General

Starting materials and reagents were obtained from commercial sources and used as-received unless otherwise specified. Dichloromethane and triethylamine were treated with calcium hydride and distilled before use. Tetrahydrofuran (THF) and diethyl ether were treated with metallic sodium and benzophenone and distilled before use. Anhydrous dimethylformamide (DMF) and pyridine were obtained from commercial sources. Anhydrous reactions were carried out with continuous stirring under an atmosphere of nitrogen. Progress of the reactions was monitored by thin-layer chromatography (TLC) analysis (silica gel 60 F254 on aluminum plates). ^1H NMR and ^{13}C NMR were recorded on 250, 400 or 500 MHz equipment, the chemical shifts (δ) were reported in parts per million (ppm) relative to the deuterated solvent as the internal standard (CDCl_3 7.26 ppm, 77.0 ppm), and coupling constants (J) are in hertz (Hz). Mass spectra were recorded on a Q-ToF apparatus operating in electrospray mode (ES). The principal absorptions of infrared spectra with Fourier transform (FTIR) are listed in cm^{-1} . The purity of all final compounds was higher than 95% and was determined by HPLC analysis, using a C18 column (4.6×150 mm, particle size $5 \mu\text{m}$) and a mixture of $\text{MeOH}/\text{H}_2\text{O}$ or $\text{MeCN}/\text{H}_2\text{O}$ as the eluent. The IUPAC names of the compounds were generated using ChemBioDraw Ultra 13.0. NMR spectra were processed using ACD/NMR Processor Academic Edition version 12.01.

Synthesis

Benzyl 4-oxo-3,4-dihydropyridine-1(2H)-carboxylate (12). A solution of 4-methoxypyridine (**11**, 467 μL , 4.44 mmol, 1 eq.) in dry methanol (6.5 mL) was cooled to -78°C . Sodium borohydride (252 mg, 6.66 mmol, 1.5 eq.) was added and the mixture was stirred for 15 minutes. Benzyl chloroformate (1000 μL , 6.66 mmol, 1.5 eq.) was dissolved in dry diethyl ether (10 mL) and added dropwise to the reaction mixture. The resulting suspension was stirred at -78°C for 3 h. The reaction was quenched by addition of water (12 mL), warmed to room temperature and stirred for 1 h. The mixture was extracted with ethyl acetate (3×10 mL). The combined organic layers were washed with brine (10 mL), dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The crude was purified by flash column chromatography, eluting with hexane/ethyl acetate (60:40). Yield: 700 mg, 68% as a colorless oil; $R_f = 0.50$, hexane/ethyl acetate (60:40); ^1H NMR (250 MHz; CDCl_3): δ 2.50 (2H, t, $J = 7.6$ Hz), 3.99 (2H, t, $J = 7.6$ Hz), 5.22 (2H, s), 5.29 (1H, d, $J = 8.2$), 7.32–7.37 (5H, m), 7.81 (1H, d, $J = 8.2$ Hz); ^{13}C NMR

(62.9 MHz; CDCl_3): δ 35.3 (CH_2), 42.3 (CH_2), 68.7 (CH_2), 107.4 (CH), 128.2 (CH), 128.44 (CH), 128.48 (CH), 134.7 (C_0), 143.1 (CH), 152.3 (C_0), 192.9 (C_0).

2,3-Dihydropyridin-4(1H)-one (13). A mixture of benzyl 4-oxo-3,4-dihydropyridine-1(2H)-carboxylate (**12**, 705 mg, 3.0 mmol, 1 eq.) and Pd/C (70 mg, 5% w/w, 0.02 eq.) in EtOH (30 mL) was stirred under an atmosphere of hydrogen at room temperature for 1.5 h. The product was filtered through a plug of celite, washed with ethyl acetate and concentrated under reduced pressure. Yield: 296 mg, quant. as a brown oil; $R_f = 0.13$, ethyl acetate; ^1H NMR (250 MHz): δ 2.51 (2H, t, $J = 7.6$ Hz), 3.60 (2H, t, $J = 7.9$ Hz), 5.05 (1H, d, $J = 7.4$ Hz), 5.48 (1H, br. s), 7.20–7.27 (1H, m); ^{13}C NMR (62.9 MHz; CDCl_3): δ 35.5 (CH_2), 41.3 (CH_2), 97.6 (CH), 153.0 (CH), 192.8 (C_0).

(E)-1-(3-(3,4,5-Trimethoxyphenyl)acryloyl)-2,3-dihydropyridin-4(1H)-one (2). (E)-3-(3,4,5-Trimethoxyphenyl)acrylic acid (118 mg, 0.49 mmol, 1.6 eq.) was dissolved in dichloromethane (3 mL) and three drops of dry DMF were added. Oxalyl chloride (41.8 μL , 0.49 mmol, 1.6 eq.) was slowly added (gas evolution!) and the reaction mixture was stirred for 4 h at room temperature. The resulting solution was added to a solution of 2,3-dihydropyridin-4(1H)-one (**13**, 30 mg, 0.31 mmol, 1 eq.), triethylamine (129 μL , 0.93 mmol, 3 eq.) and DMAP (3.81 mg, 0.03 mmol, 0.1 eq.) in dry dichloromethane (3 mL), and the resulting mixture was stirred overnight at room temperature. The reaction was quenched by washing with a saturated aqueous NH_4Cl solution (10 mL). After separation of the layers, the aqueous layer was extracted with dichloromethane (3×10 mL). The combined organic layers were dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The crude was purified by flash column chromatography, eluting with hexane/ethyl acetate (30:70). Yield: 35 mg, 36% as a white solid; $R_f = 0.25$, hexane/ethyl acetate (30:70); mp 139 – 143°C ; IR (ATR, cm^{-1}): 2945, 2918, 1650, 1611, 1581, 1291, 1155, 1125, 980; ^1H NMR (250 MHz): δ 2.63 (2H, t, $J = 6.9$ Hz), 3.90 (9H, s), 4.18 (2H, t, $J = 6.8$ Hz), 5.45 (1H, d, $J = 8.2$ Hz), 6.79 (2H, s), 6.82 (1H, d, $J = 15.1$ Hz), 7.75 (1H, d, $J = 15.1$ Hz), 7.95 (1H, d, $J = 8.1$ Hz); ^{13}C NMR (62.9 MHz; CDCl_3): δ 35.9 (CH_2), 42.3 (CH_2), 56.2 (CH_3), 61.0 (CH_3), 105.6 (CH), 108.2 (CH), 113.6 (CH), 129.6 (C_0), 140.7 (C_0), 142.8 (CH), 147.1 (CH), 153.5 (C_0), 165.2 (C_0), 193.4 (C_0); HRMS (ESI +) m/z : calcd. for $\text{C}_{17}\text{H}_{19}\text{NO}_5\text{Na}^+$ [$\text{M} + \text{Na}$] $^+$ 340.1155, found 340.1155.

1-Acetyl-2,3-dihydropyridin-4(1H)-one (7). To a solution of 2,3-dihydropyridin-4(1H)-one (**13**, 30.1 mg, 0.31 mmol, 1 eq.) in dry dichloromethane (3 mL) were added triethylamine (130 μL , 0.93 mmol, 3 eq.) and DMAP (3.8 mg, 0.03 mmol, 0.1 eq.). Acetic anhydride (44 μL , 0.46 mmol, 1.5 eq.) was added dropwise to the reaction mixture which was stirred overnight at room temperature. The reaction was quenched with a saturated aqueous NH_4Cl solution (10 mL). After separation of the layers, the aqueous layer was extracted with dichloromethane (3×10 mL). The combined organic layers were dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The crude was purified by

flash column chromatography, eluting with hexane/ethyl acetate (50:50). Yield: 33.8 mg, 78% as a white solid; R_f = 0.20, hexane/ethyl acetate (30:70); mp 57–60 °C; ^1H NMR (250 MHz): δ 2.30 (3H, s), 2.54 (2H, t, J = 7.1 Hz), 4.04 (2H, t, J = 6.8 Hz), 5.32–5.35 (1H, m), 7.45–8.33 (1H, m); ^{13}C NMR (62.9 MHz; CDCl_3): δ 21.2 (CH_3), 35.6 (CH_2), 40.3 (CH_2), 107.7 (CH), 143.0 (CH), 168.8 (C_0), 193.3 (C_0).

1-Benzoyl-2,3-dihydropyridin-4(1H)-one (8). To a solution of 2,3-dihydropyridin-4(1H)-one (13, 30.1 mg, 0.31 mmol, 1 eq.) in dry dichloromethane (3 mL) were added triethylamine (130 μL , 0.93 mmol, 3 eq.) and DMAP (3.8 mg, 0.03 mmol, 0.1 eq.). Benzoyl chloride (40 μL , 0.34 mmol, 1.1 eq.) was added dropwise to the reaction mixture and the resulting mixture was stirred overnight at room temperature. The reaction was quenched by washing with saturated aqueous NH_4Cl solution (10 mL). After separation of the layers, the aqueous layer was extracted with dichloromethane (3 $\times$ 10 mL). The combined organic layers were dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The crude was purified by flash column chromatography, eluting with hexane/ethyl acetate (30:70). Yield: 32 mg, 51% as a colorless oil; R_f = 0.28, hexane/ethyl acetate (60:40); ^1H NMR (250 MHz; CDCl_3): δ 2.67 (2H, t, J = 7.1 Hz), 4.17 (2H, t, J = 7.3 Hz), 5.33 (1H, d, J = 8.2 Hz), 7.46–7.62 (6H, m); ^{13}C NMR (62.9 MHz; CDCl_3): δ 36.0 (CH_2), 42.9 (CH_2), 107.9 (CH), 128.6 (CH), 128.8 (CH), 131.9 (CH), 132.6 (C_0), 144.8 (CH), 170.2 (C_0), 193.4 (C_0).

1-Cinnamoyl-2,3-dihydropyridin-4(1H)-one (9). Cinnamic acid (68.7 mg, 0.46 mmol, 1.5 eq.) was dissolved in dichloromethane (3 mL) and three drops of dry DMF were added. Oxalyl chloride (62.8 mg, 0.49 mmol, 1.6 eq.) was slowly added (gas evolution!) and the reaction mixture was stirred for 4 h at room temperature. The resulting solution was added to a solution of 2,3-dihydropyridin-4(1H)-one (30 mg, 0.31 mmol, 1 eq.), triethylamine (129 μL , 0.93 mmol, 3 eq.) and DMAP (3.81 mg, 0.03 mmol, 0.1 eq.) in dry dichloromethane (3 mL) and the resulting mixture was stirred overnight at room temperature. The reaction was quenched by washing with a saturated aqueous NH_4Cl solution (10 mL). After separation of the layers, the aqueous layer was extracted with dichloromethane (3 $\times$ 10 mL). The combined organic layers were dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The crude was purified by flash column chromatography, eluting with hexane/ethyl acetate (60:40). Yield: 29.0 mg, 41% as a white solid; R_f = 0.32, hexane/ethyl acetate (60:40); mp 113–116 °C; IR (ATR, cm^{-1}): 2950, 2907, 1660, 1625, 1589, 1290, 1178, 977, 769; ^1H NMR (250 MHz; CDCl_3): δ 2.65 (2H, t, J = 7.6 Hz), 4.19 (2H, t, J = 7.4 Hz), 5.46 (1H, d, J = 8.2 Hz), 6.97 (1H, d, J = 15.3 Hz), 7.43–7.44 (3H, m), 7.57–7.59 (2H, m), 7.86 (1H, d, J = 15.5 Hz), 7.98 (1H, d, J = 7.9 Hz); ^{13}C NMR (62.9 MHz; CDCl_3): δ 35.9 (CH_2), 42.3 (CH_2), 108.3 (CH), 114.6 (CH), 128.2 (CH), 129.0 (CH), 130.8 (CH), 134.2 (C_0), 142.7 (CH), 147.1 (CH), 165.2 (C_0), 193.4 (C_0); HRMS (ESI +) m/z : calcd. for $\text{C}_{14}\text{H}_{14}\text{NO}_2^+$ [$\text{M} + \text{H}$] $^+$ 228.1019, found 228.1009.

1-(3-(3,4,5-Trimethoxyphenyl)propanoyl)-2,3-dihydropyridin-4(1H)-one (10). 3-(3,4,5-Trimethoxyphenyl)propanoic acid (79.3 mg, 0.33 mmol, 1.25 eq.) was dissolved in dichloromethane (3 mL) and three drops of dry DMF were added. Oxalyl chloride (41.9 μL , 0.33 mmol, 1.25 eq.) was slowly added (gas evolution!) and the reaction mixture was stirred for 4 h at room temperature. The resulting solution was added to a solution of 2,3-dihydropyridin-4(1H)-one (25.6 mg, 0.264 mmol, 1 eq.), triethylamine (110 μL , 0.79 mmol, 3 eq.) and DMAP (3.2 mg, 0.02 mmol, 0.1 eq.) in dry dichloromethane (3 mL), and the resulting mixture was stirred overnight at room temperature. The reaction was quenched by washing with a saturated aqueous NH_4Cl solution (10 mL). After separation of the layers, the aqueous layer was extracted with dichloromethane (3 $\times$ 10 mL). The combined organic layers were dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The crude was purified by flash column chromatography, eluting with hexane/ethyl acetate (30:70). Yield: 34 mg, 40% as a colorless oil; R_f = 0.23, hexane/ethyl acetate (30:70); IR (ATR, cm^{-1}): 2997, 2942, 2841, 1663, 1590, 1298, 1181, 1124, 1007, 749; ^1H NMR (250 MHz; CDCl_3): δ 2.53 (2H, t, J = 7.1 Hz), 2.85 (2H, t, J = 6.8 Hz), 2.97 (2H, t, J = 6.8 Hz), 3.81 (3H, s), 3.84 (6H, s), 4.05 (2H, br. s), 5.35 (1H, br. s), 6.43 (2H, s), 8.70–7.3 (1H, m); ^{13}C NMR (62.9 MHz; CDCl_3): δ 31.1 (CH_2), 35.4 (CH_2), 35.7 (CH_2), 41.0 (CH_2), 56.1 (CH_3), 60.8 (CH_3), 105.4 (CH), 108.0 (CH), 136.0 (C_0), 136.6 (C_0), 142.0 (CH), 153.3 (C_0), 170.7 (C_0), 193.2 (C_0); HRMS (ESI +) m/z : calcd. for $\text{C}_{17}\text{H}_{21}\text{NO}_5\text{Na}^+$ [$\text{M} + \text{Na}$] $^+$ 342.1312, found 342.1307.

Cenocladamide (1). (*E*)-3-(4-Acetoxy-3,5-dimethoxyphenyl)-acrylic acid (91 mg, 0.34 mmol, 1.25 eq.) was dissolved in dichloromethane (3 mL) and three drops of dry DMF were added. Oxalyl chloride (27.7 μL , 0.33 mmol, 1.2 eq.) was slowly added (gas evolution!) and the reaction mixture was stirred for 4 h at room temperature. The resulting solution was added to a solution of 2,3-dihydropyridin-4(1H)-one (13, 26.5 mg, 0.27 mmol, 1 eq.), triethylamine (114 μL , 0.82 mmol, 3 eq.) and DMAP (3.4 mg, 0.03 mmol, 0.1 eq.) in dry dichloromethane (3 mL), and the resulting mixture was stirred overnight at room temperature. The reaction was quenched by washing with a saturated aqueous NH_4Cl solution (10 mL). After separation of the layers, the aqueous layer was extracted with dichloromethane (3 $\times$ 10 mL). The combined organic layers were dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The crude was purified by flash chromatography, eluting with hexane/ethyl acetate (30:70), and amide 14 was used in the next reaction. Thus, amide 14 was taken up in a mixture of $\text{MeOH}/\text{H}_2\text{O}/\text{THF}$ (4.5 mL, 4:1:4) and NH_4OAc (171 mg, 2.22 mmol, 8.1 eq.) was added in three portions over three days. After 72 h, the mixture was concentrated *in vacuo* and cenocladamide was purified by flash column chromatography, eluting with hexane/ethyl acetate (50:50). Yield: 15 mg, 18% (2 steps) as a yellowish oil; R_f = 0.20, hexane/ethyl acetate (50:50); IR (ATR, cm^{-1}): 2962, 2917, 2850, 1710, 1650, 1510, 1427, 1222, 1120; ^1H NMR (250 MHz; CDCl_3): δ 2.65

(2H, t, $J = 7.3$ Hz), 3.96 (6H, s), 4.20 (2H, t, $J = 7.6$ Hz), 5.46 (1H, d, $J = 8.4$ Hz), 5.87 (1H, br. s), 6.78 (1H, d, $J = 15.3$ Hz), 6.82 (2H, s), 7.77 (1H, d, $J = 15.2$ Hz), 7.99 (1H, d, $J = 8.2$ Hz); ^{13}C NMR (62.9 MHz; CDCl_3): δ 36.0 (CH_2), 42.4 (CH_2), 56.4 (CH_3), 105.4 (CH), 108.1 (CH), 112.0 (CH), 125.7 (C_0), 137.8 (C_0), 142.9 (CH), 147.3 (C_0), 147.6 (CH), 165.4 (C_0), 193.5 (C_0); HRMS (ESI +) m/z : calcd. for $\text{C}_{16}\text{H}_{17}\text{NO}_5\text{Na}^+$ [$\text{M} + \text{Na}$] $^+$ 326.0999, found 326.0995.

(*E*)-3-(4-Hydroxy-3,5-dimethoxyphenyl)-1-(piperidin-1-yl)prop-2-en-1-one (5). To a solution of piperidine (15, 21.6 μL , 0.219 mmol, 1 eq.) in anhydrous THF (5 mL) were added sinapic acid (50.0 mg; 0.219 mmol, 1 eq.) and DCC (45.6 mg; 0.219 mmol, 1 eq.). The mixture was stirred at room temperature for 24 h. The solvent was removed and the crude was purified by flash column chromatography, eluting with hexane/ethyl acetate (60:40). Yield: 37 mg, 58% as a white solid; $R_f = 0.58$, hexane/ethyl acetate (60:40); mp 126–128 $^\circ\text{C}$; IR (ATR, cm^{-1}): 2929, 2849, 1639, 1582, 1462, 1337, 1113; ^1H NMR (500 MHz; CDCl_3): δ 1.61–1.68 (6H, m), 3.60–3.68 (4H, m), 3.92 (6H, s), 5.82 (1H, br. s), 6.72 (1H, d, $J = 15.4$ Hz), 6.75 (2H, s), 7.56 (1H, d, $J = 15.4$ Hz); ^{13}C NMR (62.9 MHz; CDCl_3): δ 24.6 (CH_2), 25.6 (CH_2), 27.0 (CH_2), 43.3 (CH_2), 46.9 (CH_2), 56.3 (CH_3), 104.7 (CH), 115.3 (CH), 126.9 (C_0), 136.4 (C_0), 142.6 (CH), 147.1 (C_0), 165.4 (C_0).

(*E*)-1-(Piperidin-1-yl)-3-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (6). To a solution of (*E*)-3-(3,4,5-trimethoxyphenyl)acrylic acid (120 mg, 0.504 mmol, 1 eq.) in dry dichloromethane (5 mL) were added EDCI (115 mg, 0.6 mmol, 1.2 eq.), DMAP (74.0 mg; 0.6 mmol, 1.2 eq.) and triethylamine (84 μL , 0.6 mmol, 1.2 eq.). The mixture was stirred at room temperature for 30 minutes. Then, piperidine (15) was added (51.1 mg; 0.6 mmol, 1.2 eq.) and the reaction mixture was stirred at room temperature for 24 h. Dichloromethane (10 mL) was added and the reaction mixture was washed with an aqueous HCl solution (1 M, 30 mL), a saturated aqueous NaHCO_3 solution (30 mL) and brine (30 mL). The organic layer was dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The crude was purified by flash column chromatography, eluting with hexane/ethyl acetate (50:50). Yield: 81.4 mg, 53% as a yellow oil; $R_f = 0.19$, hexane/ethyl acetate (50:50); ^1H NMR (250 MHz; CDCl_3): δ 1.57–1.60 (6H, m), 3.58 (4H, br. s), 3.82 (3H, s), 3.85 (6H, s), 6.70 (2H, s), 6.75 (1H, d, $J = 15.5$ Hz), 7.50 (1H, d, $J = 15.5$ Hz); ^{13}C NMR (62.9 MHz; CDCl_3): δ 24.4 (CH_2), 43.2 (CH_2), 46.9 (CH_2), 56.0 (CH_3), 60.7 (CH_3), 104.7 (CH), 116.8 (CH), 130.9 (C_0), 139.2 (C_0), 142.0 (CH), 153.2 (C_0), 165.1 (C_0).

(*E*)-3-(4-Hydroxy-3,5-dimethoxyphenyl)-1-(1,4-dioxo-8-azaspiro[4.5]decan-8-yl)prop-2-en-1-one (17). To a solution of (*E*)-1-(1,4-dioxo-8-azaspiro[4.5]decan-8-yl)-3-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (16, 57.6 μL , 0.440 mmol, 1 eq.) in anhydrous THF (10 mL) were added sinapic acid (101 mg, 0.440 mmol, 1 eq.) and DCC (91.7 mg, 0.440 mmol, 1 eq.). The mixture was stirred at room temperature for 24 h. The solvent was removed and the crude was purified by flash column chromatography, eluting with hexane/ethyl acetate (30:70). Yield: 113 mg, 73% as a yellow oil; $R_f = 0.28$, hexane/

ethyl acetate (30:70); IR (ATR, cm^{-1}) 2964, 2876, 2841, 1639, 1601, 1515, 1334, 1223, 1116, 985; ^1H NMR (250 MHz, CDCl_3): δ 1.68–1.73 (4H, m), 3.73 (4H, br. s), 3.85 (6H, s), 3.94 (4H, s), 6.71 (2H, s), 6.72 (1H, d, $J = 15.2$ Hz), 7.54 (1H, d, $J = 15.3$ Hz); ^{13}C NMR (62.9 MHz; CDCl_3): δ 34.8 (CH_2), 35.3 (CH_2), 40.1 (CH_2), 43.5 (CH_2), 56.2 (CH_3), 64.3 (CH_2), 104.7 (CH), 106.8 (C_0), 114.5 (CH), 126.4 (C_0), 136.5 (C_0), 143.2 (CH), 147.1 (C_0), 165.5 (C_0); HRMS (ESI +) m/z : calcd. for $\text{C}_{18}\text{H}_{24}\text{NO}_6^+$ [$\text{M} + \text{H}$] $^+$ 350.1598, found 350.1586.

(*E*)-1-(1,4-Dioxo-8-azaspiro[4.5]decan-8-yl)-3-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (18). To a solution of (*E*)-3-(3,4,5-trimethoxyphenyl)acrylic acid (120 mg, 0.504 mmol, 1 eq.) in dry dichloromethane (5 mL) were added EDCI (115 mg, 0.6 mmol, 1.2 eq.), DMAP (74.0 mg; 0.6 mmol, 1.19 eq.) and triethylamine (83.6 μL , 0.6 mmol, 1.2 eq.). The mixture was stirred at room temperature for 30 minutes. Next, 1,4-dioxo-8-azaspiro[4.5]decan-8-yl (16) was added (78.5 μL ; 0.6 mmol, 1.2 eq.) and the reaction mixture was stirred at room temperature for 24 h. Then, dichloromethane (10 mL) was added and the reaction mixture was washed with an aqueous HCl solution (1 M, 30 mL), a saturated aqueous NaHCO_3 solution (30 mL) and brine (30 mL). The organic layer was dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The crude was purified by flash column chromatography, eluting with hexane/ethyl acetate (50:50). Yield: 57 mg, 31% as a white solid; $R_f = 0.27$, hexane/ethyl acetate (30:70); mp 148–149 $^\circ\text{C}$; IR (ATR, cm^{-1}): 2972, 2942, 2873, 1641, 1585, 1334, 1126; ^1H NMR (250 MHz; CDCl_3): δ 1.68–1.72 (4H, m), 3.72 (4H, br. s), 3.82 (3H, s), 3.85 (6H, s), 3.95 (4H, s), 6.70 (2H, s), 6.76 (1H, d, $J = 15.3$), 7.52 (1H, d, $J = 15.3$); ^{13}C NMR (62.9 MHz; CDCl_3): δ 34.6 (CH_2), 35.7 (CH_2), 40.2 (CH_2), 43.8 (CH_2), 56.0 (CH_3), 60.7 (CH_3), 64.3 (CH_2), 104.9 (CH), 106.7 (C_0), 116.3 (CH), 130.7 (C_0), 139.4 (C_0), 142.6 (CH), 153.2 (C_0), 165.2 (C_0); HRMS (ESI +) m/z : calcd. for $\text{C}_{19}\text{H}_{25}\text{NO}_6\text{Na}^+$ [$\text{M} + \text{Na}$] $^+$ 386.1574, found 386.1558.

(*E*)-1-(3-(4-Hydroxy-3,5-dimethoxyphenyl)acryloyl)piperidin-4-one (3). A solution of (*E*)-3-(4-hydroxy-3,5-dimethoxyphenyl)-1-(1,4-dioxo-8-azaspiro[4.5]decan-8-yl)prop-2-en-1-one (17, 56 mg, 0.16 mmol, 1 eq.) and *p*-toluenesulfonic acid monohydrate (2.4 mg, 0.013 mmol, 0.08 eq.) in a water-ethanol mixture (3 mL, 1:2) was heated at reflux for 3 h. The solvent was removed *in vacuo* and the residue was added to a saturated aqueous NaHCO_3 solution (20 mL), then extracted with ethyl acetate (20 mL) and washed with brine (20 mL). The organic layer was dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The crude was purified by flash column chromatography, eluting with hexane/ethyl acetate (30:70). Yield: 30 mg, 63% as a yellowish solid; $R_f = 0.30$, hexane/ethyl acetate (30:70); mp 192–203 $^\circ\text{C}$; IR (ATR, cm^{-1}): 2961, 2918, 2849, 1712, 1647, 1511, 1427, 1225, 1123; ^1H NMR (250 MHz, CDCl_3): δ 2.54 (4H, t, $J = 6.1$ Hz), 3.92 (6H, s), 3.97 (4H, t, $J = 6$ Hz), 5.86 (1H, br. s), 6.77 (2H, s), 6.78 (1H, d, $J = 15.3$ Hz), 7.65 (1H, d, $J = 15.1$ Hz); ^{13}C NMR (62.9 MHz; CDCl_3): δ 41.1 (CH_2), 44.1 (CH_2), 56.4 (CH_3), 105.0 (CH), 113.8 (CH), 126.3 (C_0), 136.9 (C_0), 144.4 (CH),

147.2 (C₀), 166.0 (C₀), 206.8 (C₀); HRMS (ESI +) *m/z*: calcd. for C₁₆H₁₉NO₅Na⁺ [M + Na]⁺ 328.1155, found 328.1151.

(*E*)-1-(3-(3,4,5-trimethoxyphenyl)acryloyl)piperidin-4-one (4). A solution of (*E*)-1-(1,4-dioxo-8-azaspiro[4.5]decan-8-yl)-3-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (18, 57.0 mg; 0.16 mmol, 1 eq.) and *p*-toluenesulfonic acid monohydrate (2.4 mg, 0.013 mmol, 0.08 eq.) in a water–ethanol mixture (3 mL, 1:2) was heated at reflux for 3 h. The solvent was removed *in vacuo* and the residue was added to a saturated aqueous NaHCO₃ solution (20 mL), extracted with ethyl acetate (20 mL), and the organic phase was washed with brine (20 mL), dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure to furnish the product. Yield: 37 mg, 74% as a white solid; *R*_f = 0.24, hexane/ethyl acetate (30:70); mp 100–106 °C; IR (ATR, cm⁻¹): 2991, 2969, 1716, 1649, 1585, 1416, 1268, 1125, 1002, 829; ¹H NMR (400 MHz; CDCl₃): δ 2.52 (4H, t, *J* = 6.4 Hz), 3.85 (3H, s), 3.87 (6H, s), 3.95 (4H, t, *J* = 6.0 Hz), 6.73 (2H, s), 6.80 (1H, d, *J* = 15.3 Hz), 7.63 (1H, d, *J* = 15.3 Hz); ¹³C NMR (101 MHz; CDCl₃): δ 41.5 (CH₂), 44.5 (CH₂), 56.4 (CH₃), 61.2 (CH₃), 105.4 (CH), 115.7 (CH), 130.7 (C₀), 140.1 (C₀), 144.2 (CH), 153.6 (C₀), 166.0 (C₀), 207.0 (C₀); HRMS (ESI +) *m/z*: calcd. for C₁₇H₂₂NO₅⁺ [M + H]⁺ 320.1492, found 320.1479.

Biology

Cell culture. The breast cancer cell lines MDA-MB-231, MDA-MD-468, BT549, HCC38, HS578T, MCF7, SKBR3, T47D and MCF-10A were purchased from the American Type Culture Collection (ATCC). The human mammary primary cells (HMEC) were purchased from Lonza and immortalized by stable transduction with the retroviral vector pBABE-hygro-hTERT (Telomere Reverse Transcriptase). pBABE-hygro-hTERT was a gift from Bob Weinberg (Addgene plasmid # 1773).³³ All tumor cell lines were cultivated in RPMI 1640 media (Sigma) supplemented with 10% fetal bovine serum (Vitrocell). MCF-10A cells were cultivated in F12/DMEM supplemented with 5% horse serum, 20 ng mL⁻¹ epithelial growth factor (EGF), 0.1 μg mL⁻¹ cholera toxin, 10 μg mL⁻¹ bovine insulin, and 0.5 μg mL⁻¹ hydrocortisone. iHMEC cells were grown in serum-free mammary epithelial growth medium (MEGM) supplemented with a proprietary combination of hormones (BulletKit) from Lonza. All cells were grown at 37 °C in a 5% CO₂ humidified incubator. Cells used for the *in vitro* assays were 90–100% viable as assessed by Trypan blue (Sigma) staining.

Proliferation assay. Three thousand cells were plated in a 96-well plate format and allowed to adhere overnight. In the following day, cells were treated in a single dose (20 μM) or in a serial dilution (from 1.5–50 μM) of the compounds for 72 hours. The number of cells at 24 hours was measured and considered as plated cells (Media^{24h}). Media was replaced once after 48 hours. DMSO (0.5%) or 1 μM doxorubicin was used as negative and positive control, respectively. Cells were fixed with 3.7% formaldehyde and stained with 0.4 μg mL⁻¹ DAPI (Sigma-Aldrich). The total number of DAPI-stained nu-

clei was quantified using the plate-reader fluorescence microscope Operetta (PerkinElmer) and the software Columbus (Perkin Elmer). The percentage of proliferation (when the number of cells at time 72 h of treatment > time 24 h) was calculated with the equation $100 \times [(Compound^{72h}/Media^{24h})/(DMSO^{72h}/Media^{24h})]$, while the percentage of cell loss (when the number of cells at time 72 h of treatment < time 24 h) was calculated with the equation $100 \times [1 - (Compound^{72h}/Media^{24h})]$.

Nuclear fragmentation and mitochondrial fluorescence microscopy assays. Nuclei and mitochondria were stained with 1 μg mL⁻¹ Hoechst 33342 and 300 nM MitoTracker Deep Red (Life Technologies), respectively, in RPMI and 10% fetal bovine serum for 60 minutes and imaged with the plate-reader fluorescence microscope Operetta (PerkinElmer). The software Columbus was used to identify the nuclear and cytoplasmic compartments and the average of intensity of MitoTracker staining was determined at the cytoplasm compartment and defined as the mitochondrial mass. For nuclear fragmentation index analysis, we used the Harmony (Perkin Elmer) “Apoptosis-1” module imported to the Columbus from the Ready-Made Solution (RMS) collection.

Cell cycle fluorescence microscopy assay. After 72 h of 0.2–25 μM compound 3 treatment, MDA-MB-231 cells were labelled with 30 μM EdU for 30 minutes. After that, cells were fixed with 3.7% paraformaldehyde in PBS at room temperature for 20 minutes. Cells were then washed twice in PBS and permeabilized with 0.1% Triton X100 in PBS for 15 minutes at room temperature. The incorporated EdU was labeled with AF488 using the Click-iT EdU labeling kit (Life Technologies) as guided by the fabricant. Following blocking for 30 minutes with 3% BSA in PBS, cells were incubated with 1:250 anti-pHH3 (S10) primary antibody (Cell Signaling Technologies) diluted in TBS at room temperature for 60 minutes. Cells were washed with PBS and then incubated with 1:300 AF647 secondary antibody (LifeTechnologies) and 1 μg mL⁻¹ Hoechst 33342 in TBS at room temperature for 60 minutes. Cells were finally washed twice with 0.05% Tween20 in PBS and left in PBS until microscope analysis. The Harmony (Perkin Elmer) RMS “Cell Cycle” module was used for the analysis.

Intracellular ROS fluorescence microscopy assay. MDA-MB-231 cells were treated with 6 μM compound 3 or 20 μM compound 5 for 3 and 24 hours, with and without 3 mM of the antioxidant *N*-acetyl-L-cysteine (NAC, Sigma-Aldrich). Intracellular ROS was measured using 10 μM of the membrane permeable dye 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA, Life Technologies) for 30 minutes at 37 °C. Cells were washed with PBS and the fluorescence intensity was measured with the Operetta fluorescence microscope and analyzed with the software Columbus.

Flow cytometry toxicity assay. One million MDA-MB-231 cells were seeded in 100 mm dishes and, after 24 h, treated for 72 h with compound 3 (40 μM) or compound 5 (20 μM) or for 24 h with 2 μM tacrine (Sigma-Aldrich). To quantify live and dead cell populations, the DNA-binding dyes acridine

orange (AO, Sigma-Aldrich) and ethidium bromide (EB, Sigma-Aldrich) were used. AO is a cell-permeant nucleic acid binding dye that emits green fluorescence when bound to dsDNA and red fluorescence when bound to ssDNA or RNA. EB, on the other hand, selectively and covalently labels DNA in dead cells since it is relatively impermeant to live cells. Floating and adherent cells were collected by trypsinization, pelleted by centrifugation, washed twice with PBS and resuspended in 300 μ L PBS containing 0.2 μ M AO and 0.5 μ M EB. All samples were stained and analyzed immediately at room temperature using a FACS Count II instrument (BD Biosciences) and the FlowJo software. AO and EB were excited with a blue laser (488 nm). Emission was detected with a 525/20 nm filter (for AO) and a 635/20 nm filter (for EB). Four-parameter list mode data were acquired for analysis.

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