Bioorganic & Medicinal Chemistry 21 (2013) 5748–5753

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis and biological evaluation of 1,7,8,8a-tetrahydro-3*H*-oxazolo[3,4-*a*]pyrazin-6(5*H*)-ones as antitumoral agents

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ARTICLE INFO

Article history:
Received 14 May 2013
Revised 8 July 2013
Accepted 9 July 2013
Available online 18 July 2013

Keywords:
Palladium
Domino reaction
Bicyclic piperazinones
Thyroid cancers
Caspase-3

ABSTRACT

A series of 1,7,8,8a-tetrahydro-3*H*-oxazolo[3,4-*a*]pyrazin-6(5*H*)-ones has been synthesized by an intramolecular, palladium(II) catalyzed, aminooxygenation of alkenyl ureas, readily available from glycine allylamides as starting materials. Biological tests showed that the obtained compounds are endowed with an interesting antitumoral activity against two human thyroid cancer cell lines, namely FTC-133 and 8305C, by promoting the apoptotic pathway and DNA fragmentation.

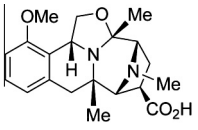
1. Introduction

Cancer has become one of the leading disease-related causes of death of human population.[1] Among all the current therapeutic methods, chemotherapy remains an important option for cancer treatment: inhibition of the characteristic proliferation pathways of cancer cells constitutes an effective strategy to fight this pathology.[2] Great research has been devoted to the discovery of new and selective anticancer agents; however, the development of drug and multidrug resistance, in which cancer cells become resistant to different structural types of chemotherapeutic agents, is one of the major hindrances to successful cancer chemotherapy.[3] Thus, the discovery and identification of novel compounds with different characteristic than the currently used or showing an alternative mechanism of action, is an intensively explored research area in medicinal chemistry.[4]

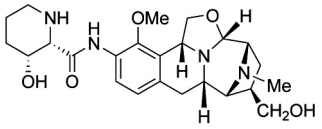
Nitrogen heterocycles are found in the majority of clinically approved drugs.[5] One of important pharmacologically relevant heterocycles is the piperazine ring system, which is found in ca. 7% of all small therapeutic molecules approved by the FDA, starting from the year 2000.

The piperazine ring is also used as both rigid-dimensional diamine core structure, as well as a modifying group to introduce an amino residue in the molecule.[6] The incorporation of a further heterocycle ring into the prospective pharmaceutical candidate (i.e. the piperazine derivative) is a well-known tactic to gain activity and safety advantages. In this context, much work has been directed towards the design and the synthesis of novel fused-piperazine derivatives.[7]

As part of our medicinal chemistry program aimed at the discovery of potential anticancer agents,[8] we have addressed our attention to the synthesis of a novel series of derivatives containing the oxazolo–pyrazine pharmacophore.[9] The oxazole unit constitutes the core structure of several biological active compounds: oxazole containing heterocycle compounds play an important role in medicinal chemistry as antimicrobial, CNS agents, antihyperglycemic



Quinocarcin



Tetrazomine

Figure 1. Structures of some anticancer agents containing the oxazolo–piperazine core.

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http://dx.doi.org/10.1016/j.bmc.2013.07.019

potentiating compounds, antitumor, analgesics and anti-inflammatory agents.[10] Quinocarcin,[11] which has shown remarkable antiproliferative activity against lymphocytic leukemia, and tetrazomine, a potent antitumoral antibiotic,[12] (Fig. 1) are two relevant examples of compounds containing an oxazolo–piperazine core.

On the other hand, small molecules targeting DNA/RNA have attracted significant scientific interest due to the medicinal, biochemical and biological implications of such molecular recognition events. The design and the synthesis of novel molecular entities, with multiple DNA/RNA binding features and with a structurally well-defined architecture, is still highly demanding.[13]

On the basis of these consideration, we report here the synthesis and the biological evaluation of oxazolo–piperazine hybrid compounds in which an oxazolo–pyrazine unit is linked to an arylimine moiety. The synthetic approach exploits the intramolecular, palladium(II) catalyzed, aminooxidation of alkenyl ureas, prepared starting from glycine allylamides. The biological effects of the new obtained compounds have been evaluated on human follicular (FTC-133) and anaplastic (8305C) thyroid carcinoma cell lines, as representatives of two aggressive types of thyroid cancer, poorly differentiated and dedifferentiated, respectively.[14–16]

2. Results and discussion

2.1. Chemistry

The preparation of the 1,7,8,8a-tetrahydro-3*H*-oxazolo[3,4-*a*]pyrazines **11a–c** and **12** is shown in Scheme 1: the key step of the synthetic process exploits a transition metal-catalyzed double intramolecular domino process.[17] Thus, the *N*-allyl-amides of Boc-glycine **4** and **5**, obtained by the reaction of *N*-Boc-glycine **1** with cyclohexyl- or phenyl-allylamine **2** and **3**, respectively, in the presence of DCC and DMAP,[18] were converted to the corresponding allyl amides **6** and **7**, by removal of the *t*-butoxycarbonyl group, and, then, treated with the appropriate commercially available or easily prepared aryl isocyanates **8a–c**,[19] respectively.

The cyclization of the alkenyl ureas **9a–c** and **10** was conveniently performed working with a $PdCl_2(MeCN)_2/CuCl_2$ catalytic system, already reported in literature as an useful tool for domino reactions of alkenyl derivatives with nucleophiles.[20] Indeed, bicyclic ring-fused piperazinones **11a–c** and **12** were achieved in high yield with $PdCl_2(MeCN)_2$ as the catalyst (5 mol %) and $CuCl_2$ (3 equiv) in THF under reflux at 60 °C for 12 h.

The structure of the obtained compounds was assigned on the basis of spectroscopic data. In particular, in the ^1H NMR spectra, chemical shifts attributable to the resonance of all protons are strictly close to those of analogous 3*H*-oxazolo[3,4-*a*]pyrazine derivatives, previously obtained by cyclization of Boc-glycine allylamides.[18] On the other hand, in the ^{13}C NMR spectra, the resonance of the methylene group of the five-membered ring, in the range 67.7–68.0 ppm, is consistent with the 4,5-dihydro-oxazole ring. These values allow to exclude an imidazolidinone ring, whose methylene group is reported to resonate in the range of 40–50 ppm.[21]

The aminooxygenation products **11a–c** and **12** are explainable by the attach of the oxygen atom on the σ-alkylcomplex **13**, generated by aminopalladation of the amides **9** and **10** (Scheme 2). In principle, according to the nucleophilic behavior of secondary amides in transition metal-catalyzed reactions,[22] both oxygen and nitrogen atoms of the urea group could be involved in the second intramolecular step, leading to the formation of an oxazole or an imidazole ring fused to the piperazinone moiety.

However, our experimental conditions have allowed a complete control of two alternative reaction pathways, with the exclusive formation of the target oxazolo–piperazine conjugates **11a–c** and **12**.

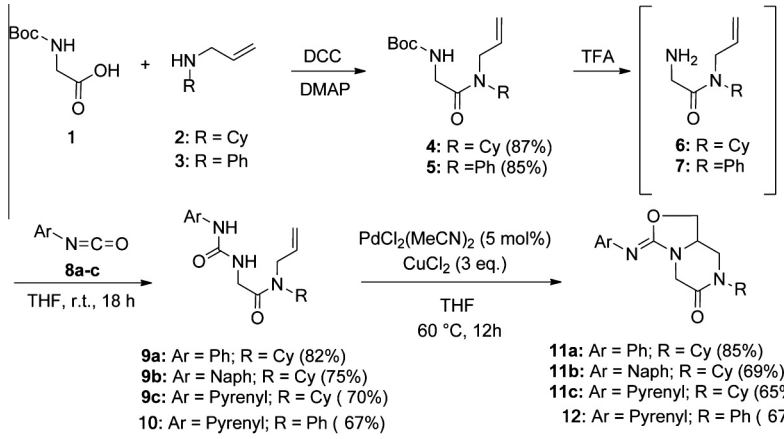
2.2. Biological evaluation

2.2.1. Cytotoxic effect of the compounds

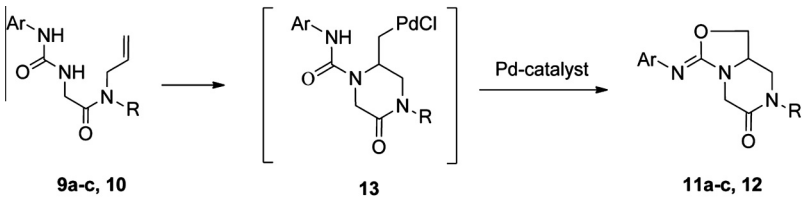
To evaluate the antitumor potential of compounds **11a–c** and **12**, we used two cancer cell lines, FTC-133 and 8305C, as representative of follicular and anaplastic thyroid human cancer, respectively. To monitor cell viability, both fluorescent microscope analysis and MTT assays have been used. In preliminary experiments, FTC-133 and 8305C cell line cultures were exposed to different concentrations (5–100 μM) of the synthesized compounds **11a–c** and **12** for 24 h, in order to establish the optimal concentration.[23–25] A significant reduction in cellular viability was observed in both FTC-133 and 8305C cell lines treated with the compounds **11a–c** and **12**, at 50 μM concentrations for 24 h, when compared with the respective untreated cell cultures used as controls. The 50% cytotoxic inhibitory concentration (IC_{50}), causing 50% decreasing in cell proliferations, obtained graphically from dose-effect curves using Prism 5.0 (GraphPad Software Inc.), is reported in Table 1. All compounds displayed cytotoxic effects on the both cell lines, at concentrations ranging from 44 to 80 μM. FTC-133 cells were more susceptible to treatment with compounds **11a–c** and **12**, than the 8305C cells.

2.2.2. Evaluation of the apoptotic pathway activation

To investigate whether apoptosis was triggered by treatment with the new compounds, caspase-3 cleavage by



Scheme 1. Synthesis of 1,7,8,8a-tetrahydro-3*H*-oxazolo[3,4-*a*]pyrazine compounds **11a–c** and **12**.



Scheme 2. Selective formation of aminooxygenation products **11a–c** and **12**.

Table 1
Concentrations (μM) of investigated compounds **11a–c**, and **12** that induced 50% decrease (IC_{50}) in FTC-133 and 8305C cell proliferation[a]

Cell lines	Untreated (control)	**11a**	**11b**	**11c**	**12**
FTC–133	>100	50.03 ± 3.90	44.79 ± 4.27	48.32 ± 2.98	74.21 ± 3.93
8305C	>100	70.66 ± 2.13	61.03 ± 5.27	68.45 ± 3.71	80.28 ± 4.52

[a] FTC–133 and 8305C cell lines were incubated with drug compounds in concentration ranging from 5 to 100 μM at 37 °C in a 5% CO_2 atmosphere for 24 h. Viability was determined by the MTT assay. Each data represents mean ± SD from four independent experiments, performed in triplicate.

immunocytochemical analysis was evaluated after 24 h of cell incubation at 50 μM concentration of compounds **11a–c** and **12**. Significant enhancement of caspase-3 positive cells was detected after 24 h of treatment in FTC-133 and 8305C cell lines, when compared to the untreated control. The effect appeared more evident in FTC-133 cell lines, with compound **12** showing the lowest activity with respect **11a–c** (Fig. 2A).

The quantification and statistical analysis of caspase-3 immunolabeling is reported in Table 2.

2.2.3. Evaluation of DNA fragmentation and molecule/DNA labeling

We tested DNA fragmentation and the eventual molecule/DNA labeling in untreated and treated FTC-133 and 8305C thyroid cancer cell lines. We observed that all the molecules tested, and prevalently **11a–c** compounds, were able to induce DNA fragmentation in treated cancer cell lines. The effect appeared more evident in FTC-133 cancer cell lines. Furthermore, to assess the molecule/DNA labeling, gel electrophoresis was performed in absence or in presence of SYBR Green I, a well-known fluorescent dye that binds specifically to double-stranded DNA (Fig. 3). Molecule/DNA labeling was detected only in presence of the fluorescent dye. The obtained data show that all the molecules induced DNA fragmentation at a different degree.

Table 2
Quantification and statistical analysis of caspase-3 positive cells in FCT–133 and 8305C human thyroid cancer cell line cultures untreated (Control) and treated with 50 μM **11a–c** or 12 for 24 h.

	% Caspase-3 positive FTC–133 cell lines	% Caspase-3 positive 8305C cell lines
Treatment	24 h	24 h
Control	2.51 ± 1	3.22 ± 1*
11a	88.68 ± 1*	75.88 ± 1*
11b	90.56 ± 2*	78.56 ± 2*
11c	89.24 ± 2*	68.30 ± 3*
12	29.30 ± 3*	18.24 ± 2*

* $p < 0.05$ versus respective control by one-way ANOVA followed by post hoc Holm–Sidak test. Data were collected from 4 fields/coverslip of four experiments in duplicate.

According to this test, our data suggest a weak stacking interaction between the compounds **11a–c**, **12**, and the DNA bases, probably due to a hydrogen bond between the amido group of the piperazine group and the amino group of poly-d(AT) and/or poly-d(GC) chain lying in the minor groove. Furthermore, the low activity observed in both cancer cell line cultures for compound **12**, which possesses a phenyl ring into the amido portion of the molecule, might be related to a reduced electron releasing effect (cross

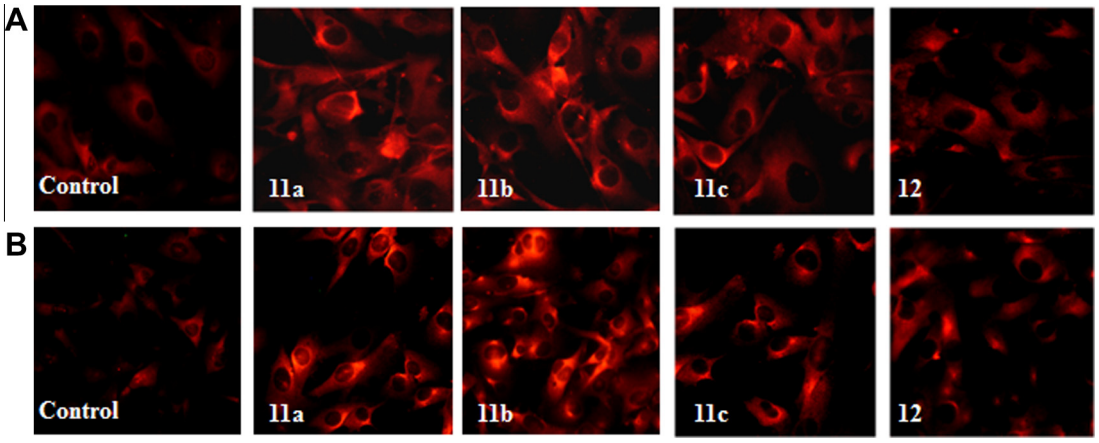


Figure 2. (A) Fluorescent microscope analysis of caspase-3 cleavage in (A) FCT-133 and (B) 8305C human thyroid cancer cell lines, untreated (control) and treated with 50 μM **11a–c** or **12** for 24 h. Scale bars = 50 μm.

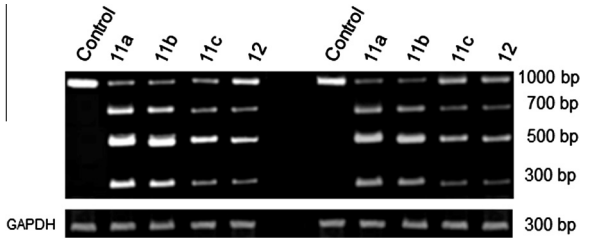


Figure 3. DNA fragmentation in untreated or treated FTC-133 (left) and 8305C (right) cells with 50 μM of **11a–c** and **12** for 24 h. Gel electrophoresis was performed in presence of SIBER Green I. Glyceraldehyde-3-phosphate dehydrogenase-1 (GAPDH-1) gene was used as a reference gene to normalize target gene expression.

conjugation) which decreases the ability to form a hydrogen bond with the DNA-nucleobases.

3. Conclusions

In summary, we report an efficient synthesis of a series of oxazolo[3,4-*a*]pyrazin-6(3*H*)-ones, according to a procedure based on a double intramolecular domino process catalyzed by $PdCl_2(MeCN)_2$/$CuCl_2$ performed on different alkenyl ureas. Biological tests indicate that the obtained compounds are endowed with an interesting antitumor activity against two thyroid carcinoma cell lines, by activating caspase-3 dependent apoptotic pathway and DNA fragmentation with a prevalent action on follicular type.

4. Experimental section

Flash column chromatography was performed employing 230–400 mesh silica gel. Analytical thin layer chromatography was performed on silica gel 60 F_{254}. Melting points were measured with a Büchi B-540 apparatus and are uncorrected. IR spectra were recorded on a Nicolet 550 FT-IR spectrophotometer. Nuclear magnetic resonance spectra were acquired on Varian instrument at 200 or 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR. ^{13}C spectra were ^{1}H decoupled and multiplicities were determined by the APT pulse sequence. EI mass spectra were recorded at an ionizing voltage of 6 KeV on a VG 70-70 EQ. Elemental analyses were executed on a Perkin–Elmer CHN Analyzer Series II 2400.

4.1. General procedure for the preparation of alkenyl ureas 9a–c and 10

Trifluoroacetic acid (3 mL) was added to a solution of allyl amide **4** or **5** (1 mmol) in CH_2Cl_2 (3 mL). The mixture was stirred at room temperature for 1 h. The solvent was evaporated to dryness and the residue was taken with a 1 M solution of NaOH up to pH 13. The mixture was extracted with CH_2Cl_2 (3 × 10 mL) and the organic layer was dried over Na_2SO_4. Concentration at reduced pressure gave a colourless oil, which was dissolved in THF (5 mL) and treated with the appropriate isocyanate **8a–c** (1 mmol). The solution was stirred at room temperature overnight and then the solvent was evaporated to give a crude residue that was chromatographed on silica gel column.

4.1.1. *N*-Allyl-*N*-cyclohexyl-2-(3-phenylureido)-acetamide (9a)

Eluent: light petroleum–AcOEt 3:2. White solid; yield 82%, mp 97–99 °C. IR (nujol): ν = 3301, 1750, 1654 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$, mixture of two rotamers in 7:6 ratio) major rotamer: δ 1.07–1.82 (m, 10H), 3.91 (d, J = 4.8 Hz, 2H), 4.13 (s, 2H), 4.34–4.46 (m, 1H), 5.06–5.26 (m, 2H), 5.72–5.87 (m, 1H), 6.45 (br s, 1H), 7.00–7.03 (m, 1H), 7.24–7.28 (m, 2H), 7.32–7.36 (m, 2H) 7.64 (br s, 1H); minor rotamer: δ 1.07–1.82 (m, 10H), 3.47–3.59 (m, 1H), 3.94 (d, J = 5.1 Hz, 2H), 4.23 (s, 2H), 5.06–5.26 (m, 2H), 5.72–5.87 (m, 1H), 6.45 (br s, 1H), 7.00–7.03 (m, 1H), 7.24–7.28 (m, 2H), 7.32–7.36 (m, 2H), 7.64 (br s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) major rotamer: δ 25.3, 25.5, 29.7, 44.7, 46.1, 52.4, 117.5, 118.5, 121.1, 128.8, 129.1, 137.7, 155.5, 164.4; minor rotamer: δ 25.3, 25.4, 29.3, 44.5, 46.1, 47.6, 116.8, 118.5, 121.1, 128.8, 129.1, 138.3, 155.6, 164.4. MS: m/z 315 (M^+). Anal. Calcd for $C_{18}H_{25}N_3O_2$: C, 68.54; H, 7.99; N, 13.32. Found C, 68.72; H, 7.73; N, 13.41.

4.1.2. *N*-Allyl-*N*-cyclohexyl-2-(3-naphthalen-1-ylureido)-acetamide (9b)

Eluent: light petroleum–AcOEt 3:2. Beige solid; yield 75%, mp 173–174 °C. IR (nujol): ν = 3287, 1745, 1656 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$, mixture of two rotamers in 4:3 ratio) major rotamer: δ 1.02–1.79 (m, 10H), 3.74 (d, J = 4.8 Hz, 2H), 4.11 (s, 2H), 4.19–4.32 (m, 1H), 4.98–5.16 (m, 2H), 5.56–5.72 (m, 1H), 6.73 (br s, 1H), 7.42–7.50 (m, 3H), 7.63 (br s, 1H), 7.82–7.86 (m, 2H), 8.08–8.13 (m, 2H); minor rotamer: δ 1.02–1.79 (m, 10H), 3.39–3.45 (m, 1H), 3.81 (d, J = 5.4 Hz, 2H), 4.20 (s, 2H), 4.98–5.16 (m, 2H), 5.56–5.72 (m, 1H), 6.81 (br s, 1H), 7.42–7.50 (m, 3H,), 7.67 (br s, 1H), 7.82–7.86 (m, 2H), 8.08–8.13 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) major rotamer: δ 25.3, 25.5, 29.6, 42.6, 44.8, 54.3, 114.1, 116.9, 121.3, 122.0, 125.2, 125.8, 126.0, 128.3, 128.5, 134.1, 134.3, 157.0, 169.6; minor rotamer: δ 25.3, 25.4, 29.2, 42.4, 44.3, 56.4, 114.1, 116.1, 121.1, 121.9, 125.1, 125.5, 125.9, 128.3, 128.5, 133.7, 134.5, 156.9, 168.4. MS: m/z 365 (M^+). Anal. Calcd for $C_{22}H_{27}N_3O_2$: C, 72.30; H, 7.45; N, 11.50. Found C, 72.09; H, 7.61; N, 11.73.

4.1.3. *N*-Allyl-*N*-cyclohexyl-2-(3-pyren-1-ylureido)-acetamide (9c)

Eluent: light petroleum–AcOEt 3:2. Brown solid; yield 70%, mp 97–99 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.02–1.79 (m, 10H), 3.58–3.69 (m, 1H), 3.94–4.02 (m, 1H), 4.44 (d, 1H), 4.53 (d, 1H), 5.10–5.37 (m, 2H), 5.72–5.90 (m, 2H), 7.34 (br d, 1H), 8.00–8.24 (m, 9H), 8.70 (d, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 24.9, 25.8, 29.2, 29.7, 43.1, 46.0, 52.5, 118.4, 121.4, 122.8, 124.5, 124.8, 125.0, 125.3, 125.9, 126.5, 127.3, 127.5, 128.0, 129.0, 129.7, 131.0, 131.3, 131.7, 132.1, 140.1, 157.0, 169.3. MS: m/z 439 (M^+). Anal. Calcd for $C_{28}H_{29}N_3O_2$: C, 76.51; H, 6.65; N, 9.56. Found C, 76.39; H, 6.88; N, 9.59.

4.1.4. *N*-Allyl-*N*-phenyl-2-(3-pyren-1-ylureido)-acetamide (10)

Eluent: light petroleum–AcOEt 3:2. Brown solid; yield 67%, mp 103–105 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 3.81 (d, J = 4.4 Hz, 1H), 4.15 (d, J = 6.3 Hz, 2H), 4.90–4.99 (m, 2H), 5.61–5.74 (m, 1H), 6.30 (br s, 1H), 7.01–7.05 (m, 2H), 7.23–7.27 (m, 4H), 7.81 (br s, 1H), 7.98–8.05 (m, 3H), 8.11–8.30 (m, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 24.9, 25.8, 29.2, 43.1, 45.8, 52.5, 118.4, 121.2, 122.8, 124.7, 124.7, 125.0, 125.3, 126.0, 126.5, 127.2, 127.5, 128.0, 128.6, 129.8, 130.9, 131.3, 132.1, 140.2, 156.9, 169.2. MS: m/z 433 (M^+). Anal. Calcd for $C_{28}H_{23}N_3O_2$: C, 77.58; H, 5.35; N, 9.69. Found C, 77.76; H, 6.11; N, 9.92.

4.2. General procedure for synthesis of 3-arylimino-1,7,8,8a-tetrahydro-3*H*-oxazolo[3,4-*a*]pyrazin-6(5*H*)-ones 11a–c and 12

A solution of the appropriate alkenyl urea **9a–c** or **10** (1 mmol) in dry THF (3 mL) was added to a suspension of $PdCl_2(MeCN)_2$ (13 mg, 0.05 mmol) and $CuCl_2$ (402 mg, 3.0 mmol) in dry THF (3 mL). The reaction was heated at 60 °C for 12 h. After addition of brine, the mixture was extracted with CH_2Cl_2 (3 × 10 mL). The organic layer was dried over Na_2SO_4 and the solvent was

evaporated under reduced pressure to give a crude product that was purified by silica gel column chromatography.

4.2.1. 7-Cyclohexyl-3-phenylimino-1,7,8,8a-tetrahydro-3*H*-oxazolo[3,4-*a*]pyrazin-6(5*H*)-ones (11a)

Eluent: light petroleum–AcOEt 3:7. White solid; yield 85%, mp 155–156 °C. IR (nujol): ν = 1757, 1585 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 1.25–1.82 (m, 10H), 3.15 (dd, J = 11.1, 9.3 Hz, 1H), 3.28 (dd, J = 12.0, 3.8 Hz, 1H), 3.82–3.87 (m, 1H), 3.88 (d, J = 17.8 Hz, 1H), 4.02 (dd, J = 8.6, 4.4 Hz, 1H), 4.43 (dd, J = 8.6, 7.4 Hz, 1H), 4.46–4.52 (m, 1H), 4.53 (d, J = 17.8 Hz, 1H) 6.89–6.98 (m, 1H), 7.05 (d, J = 7.6 Hz, 2H), 7.20–7.24 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 25.3, 25.4, 25.6, 29.3, 29.6, 43.6, 46.4, 51.6, 52.6, 67.9, 122.5, 123.4, 128.5, 146.6, 150.9, 164.3. MS: m/z 313 (M^+). Anal. Calcd for $C_{18}H_{23}N_3O_2$: C, 68.98; H, 7.40; N, 13.41. Found C, 69.09; H, 7.17; N, 13.66.

4.2.2. 7-Cyclohexyl-3-(naphthalen-1-ylimino)-1,7,8,8a-tetrahydro-3*H*-oxazolo[3,4-*a*]pyrazin-6(5*H*)-ones (11b)

Eluent: light petroleum–AcOEt 3:7. White solid; yield 69%, mp 175–176 °C. IR (nujol): ν = 1753, 1581 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 1.05–1.82 (m, 10H), 3.24 (dd, J = 11.3, 10.6 Hz, 1H), 3.34 (dd, J = 12.0, 3.9 Hz, 1H), 3.92–3.98 (m, 1H), 4.02-4.07 (m, 2H), 4.47 (dd, J = 8.7, 7.2 Hz, 1H), 4.53–4.60 (m, 1H), 4.75 (d, J = 18.3 Hz, 1H), 7.19–7.21 (m, 1H), 7.36–7.45 (m, 3H), 7.52 (d, J = 8.2 Hz, 1H), 7.29–7.32 (m, 1H), 8.19–8.22 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 25.3, 25.4, 25.6, 29.3, 29.7, 43.6, 46.6, 51.7, 52.6, 67.8, 117.4, 122.6, 124.1, 124.9, 125.7, 125.8, 127.7, 129.3, 134.3, 142.8, 150.9, 164.4. MS: m/z 363 (M^+). Anal. Calcd for $C_{22}H_{25}N_3O_2$: C, 72.70; H, 6.93; N, 11.56. Found C, 72.63; H, 7.19; N, 11.80.

4.2.3. 7-Cyclohexyl-3-(pyrenyl-1-ylimino)-1,7,8,8a-tetrahydro-3*H*-oxazolo[3,4-*a*]pyrazin-6(5*H*)-ones (11c)

Eluent: light petroleum–AcOEt 3:7. Yellow bright solid; yield 65%, mp 132–134 °C. ^1H NMR (500 MHz, $CDCl_3$): δ 1.05–1.82 (m, 10H), 3.34 (dd, J = 11.3, 10.6 Hz, 1H), 3.40 (dd, J = 12.0, 3.9 Hz, 1H), 4.02–4.07 (m, 1H), 4.16–4.12 (m, 2H), 4.56–4.60 (m, 2H), 4.85 (d, J = 18.3 Hz, 1H), 7.19–7.21 (m, 1H), 7.36–7.45 (m, 3H), 7.52 (d, J = 8.2 Hz, 1H), 7.29–7.32 (m, 1H), 8.19–8.22 (m, 2H), 8.45 (d, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 25.3, 25.4, 25.6, 29.3, 29.7, 43.7, 46.7, 51.9, 52.6, 68.0, 120.4, 123.6, 124.1, 124.2, 125.0, 125.4, 125.5, 125.7, 126.2, 127.0, 127.4, 131.6, 142.1, 151.8, 164.4. MS: m/z 437 (M^+). Anal. Calcd for $C_{28}H_{27}N_3O_2$: C, 76.86; H, 6.22; N, 9.60. Found C, 77.05; H, 6.07; N, 9.87.

4.2.4. 7-Phenyl-3-(pyrenyl-1-ylimino)-1,7,8,8a-tetrahydro-3*H*-oxazolo[3,4-*a*]pyrazin-6(5*H*)-ones (12)

Eluent: light petroleum–AcOEt 3:7. Light yellow solid; yield 67%, mp 93–96 °C. ^1H NMR (500 MHz, $CDCl_3$): δ 3.58–3.66 (m, 2H), 3.89–3.94 (m, 1H), 4.06–4.09 (m, 2H), 4.52–4.55 (m, 2H), 5.04 (d, J = 18.8 Hz, 1H), 7.32 (d, 2H) 7.44 (t, 2H) 7.86 (d, J = 7.9 Hz 1H), 7.90–8.01 (m, 4H), 8.1–8.06 (m, 3H), 8.49 (d, J = 9.2 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 29.7, 31.6, 46.7, 46.9, 51.7, 52.8, 67.7, 96.1, 120.3, 123.6, 124.1, 124.3, 124.7, 125.0, 125.1, 125.5, 125.7, 125.8, 126.2, 126.3, 127.0, 127.4, 127.5, 127.6, 129.4, 129.5, 131.5, 131.6, 140.9, 141.9, 150.8, 165.0. MS: m/z 431 (M^+). Anal. Calcd for $C_{28}H_{21}N_3O_2$: C, 77.94; H, 4.91; N, 9.74. Found C, 78.01; H, 4.76; N, 9.48.

4.3. Biological assay

4.3.1. Materials

Dulbecco's modified Eagle medium (DMEM) and Minimum Essential Medium (MEM) containing 2 mM GlutaMAX (GIBCO), Ham's F12 (GIBCO), nonessential amino acids, heat inactivated-Foetal Bovine Serum (FBS, GIBCO), Normal Goat Serum (NGS, GIBCO), Streptomycin and penicillin antibiotics, Trypsin–EDTA 0.05% solution were from Invitrogen (Milan, Italy). Lab-Tek™ Chamber Slides II, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide salts (MTT), agarose, and other chemicals of analytical grade were obtained from Sigma–Aldrich (Milano, Italy). Mouse monoclonal antibody against caspase-3 was from Becton–Dickinson (Milan, Italy). Tetrarhodamine isothiocyanate (TRITC)-conjugated anti-mouse IgG polyclonal antibody, were from Chemicon (Prodotti Gianni, Milan, Italy). QIAamp DNA Mini kit was from Qiagen.

4.3.2. Cell cultures

FTC-133 and 8305C cell lines were suspended in appropriate medium and plated in flasks at a final density of 2×10^6 cells or in Lab-Tek™ Chamber Slides II at a final density 0.5×10^5 cells/well. Specifically the medium for FTC-133 cell lines was: DMEM containing 2 mM Gluta-MAX, 10% FBS, streptomycin (50 μg/mL), penicillin (50 U/mL); whereas the medium for 8305C cell lines was: MEM containing 2 mM Gluta-MAX, 10% FBS, streptomycin (50 μg/mL), penicillin (50 U/mL), and 1% nonessential amino acids. Cell lines were then incubated at 37 °C in humidified atmosphere containing 5% CO_2 and the medium was replaced every 2 or 3 days. When the cultures were about 85–90% confluent, cells were trypsinized by 0.05% trypsin and 0.53 mM EDTA at 37 °C in humidified atmosphere containing 5% CO_2 for 5 min. Trypsinization was stopped by adding 20% FBS, resuspended and plated in flasks fed with fresh basic complete media. Cells were seeded again at 1:4 density ratio and incubated at 37 °C in humidified atmosphere containing 5% CO_2.

4.3.3. Treatment of the cells

FTC-133 and 8305C were replated on to Lab-Tek™ Chamber Slides II at a final density of 1×10^4 cells/well, and fed in fresh complete medium. In preliminary experiments, we exposed the both cultures in the absence or the presence of different concentrations of **11a–c** or **12** (5, 10, 25, 50, 75, 100 μM) for 12, 24 h, in order to establish the optimal concentrations and their exposure times to all synthesized compounds. For this purpose, MTT test and morphological characterization were utilized.[26] We found that for the both cultures the optimal concentration of all synthesized compounds was 50 μM and the optimal exposure time was 24 h.

4.3.4. MTT bioassay

Cell survival analysis was performed by MTT reduction assay, evaluating mitochondrial dehydrogenase activity.[23,24] Cells were set up 6×10^5 cells per well of a 96-multiwell, flat-bottomed, 200 μL microplate, and maintained at 37 °C in a humidified 5% CO_2/95% air mixture.[26] At the end of treatment time, 20 μL of 0.5% MTT in (pH 7.4) PBS were added to each microwell. After 1 h of incubation with the reagent, the supernatant was removed and replaced with 200 μL of dimethyl sulfoxide (DMSO). The optical density of each well was measured with a microplate spectrophotometer reader (Titertek Multiskan; Flow Laboratories, Helsinki, Finland) at 570 nm.

4.3.5. Immunocytochemistry

Expression of caspase-3 in FTC-133 and 8305C cells was identified by immunocytochemical procedures.[24,25] Untreated or **11a–c** and **12** treated FTC-133 and 8305C were fixed by exposing to 4% paraformaldehyde in 0.1 M PBS for 20 min. Then, cells were washed three times with PBS and incubated for 1 h at 37 °C in humidified air and 5% CO_2 with 1% NGS in PBS to block unspecific sites. The cells were successively incubated overnight at 37 °C in humidified air and 5% CO_2 with mouse monoclonal antibody against caspase-3 (1:200). Finally, the slides were washed three

times with PBS, mounted in PBS/glycerol (50:50), and analyzed on a Leica fluorescent microscopy (Germany). No nonspecific staining of hMSCs was observed in control incubations in which the primary antibody was omitted.

4.3.6. DNA labelling assay

DNA extraction from both untreated and treated FTC-133 and 8305C cells with 75 μM of **11a–c** or **12** for 24 h was performed according to the user's manual. Gel electrophoresis separates DNA fragments by size in an 2% agarose gel. DNA is visualized by including in the gel an intercalating dye, SYBER Green I. GAPDH-1 as housekeeping gene was used.

4.3.7. Statistical analysis

Data were statistically analysed using one-way analysis of variance (one-way ANOVA) followed by post hoc Holm–Sidak test to estimate significant differences among groups. Data were reported as mean ± SD of four experiments in duplicate, and differences between groups were considered to be significant at $p < 0.05$.

Acknowledgments

We gratefully acknowledge the MIUR, the Universities of Catania, Messina and Insubria and CINMPIS for partial financial support.

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19. Synthesis of 1-pyrenyl isocyanate. Pyrene-1-carboxylic acid (0.5 g, 2.0 mmol), was dissolved in oxalyl chloride (5 mL) at room temperature under N_2. After stirring for 24 h the oxalyl chloride was removed in vacuo to leave a pale yellow solid. Benzene (10 mL × 2) was added and distilled in vacuo to remove residual oxalyl chloride. The resulting solid was dissolved in 20 mL of benzene, treated with trimethylsilylazide (1 mL) and refluxed for 24 h. At the end of this time the solvent was removed in vacuo to afford 1-pyrenyl isocyanate **13** (85% yield). Red brown solid, mp 290–294 °C); ^{1}H NMR ($CDCl_3$, 200 MHz): δ 7.70 (d, J = 8.3 Hz, 1H), 7.97–8.04 (m, 4H), 8.09–8.22 (m, 4H). ^{13}C NMR ($CDCl_3$, 125 MHz): 121.61, 123.0, 123.72, 124.01, 124.13, 125.05, 125.27, 125.47, 126.37, 126.66, 126.81, 126.87, 127.07, 127.19, 127.4, 128.2, 128.9, 130.9, 131.16, 131.25, 131.8, 135.8. Anal. Calcd for $C_{17}H_9NO$: C, 83.94; H, 3.73; N, 5.76. Found C, 83.89; H, 3.78; N, 5.74.
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