Unravelling the Antiproliferative Activity of 1,2,5-oxadiazole Derivatives

DANIEL EHRSAM[1†], FABIOLA PORTA[1†], MATTEO MORI[2], HENRIETTE E. MEYER ZU SCHWABEDISSEN[1], LISA DALLA VIA[3], AÌDA NELLY GARCIA-ARGAEZ[3], LIVIA BASILE[4], FIORELLA MENEGHETTI[2], STEFANIA VILLA[2]* and ARIANNA GELAIN[2]

[1]*Biopharmacy, Department of Pharmaceutical Sciences, University of Basel, Basel, Switzerland;*
[2]*Department of Pharmaceutical Sciences, University of Milan, Milan, Italy;*
[3]*Department of Pharmaceutical and Pharmacological Sciences, University of Padua, Padua, Italy;*
[4]*Department of Drug Sciences, Section of Medicinal Chemistry, University of Catania, Catania, Italy*
[†] equally contributed

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Correspondence to: Stefania Villa, Department of Pharmaceutical Sciences, University of Milan, via L. Mangiagalli 25, 20133 Milan, Italy, Phone: +390250319368, Fax: +390250319359, stefania.villa@unimi.it.

Abstract. *Background: Our efforts, focused on the discovery of novel antiproliferative agents, led to the identification of an interesting oxadiazole derivative, MD77. Aim: To complete the studies concerning the antiproliferative profile of MD77 , we developed several new derivatives. Materials and Methods: The substitution pattern around the phenyl rings of this compound was analysed through the synthesis of positional isomers and of analogues bearing different substituents at the para positions (2-12). Results: The results of the antiproliferative activity of these derivatives versus HCT-116 and HeLa cancer cell lines shed light on the effects of the presence, nature and position of such substituents. Notably, derivative 4, a regioisomer of 1 in which the substituents at the para positions of the phenyl rings were inverted, showed the best antiproliferative profile, exhibiting a significant activity also against MCF7 and MDA-MB 468 cancer cell lines. Conclusion: Preliminary results showed the ability of compound 4 to counteract human recombinant topoisomerase II α relaxation activity.*

Introduction

Cancer still ranks second behind cardiovascular diseases among the leading causes of death globally (1) and chemotherapy is the mainstay in the reduction of mortality. Nevertheless, anticancer drugs are generally extremely toxic, while their poor selectivity and the insurgence of resistance mechanisms clearly underscore the need of developing novel chemical entities, exhibiting higher efficacy and minor side effects.

During our ongoing research concerning the identification of new compounds endowed with anticancer activity (2-11), we investigated the properties of 1,2,5-oxadiazole derivatives. Among them, MD77 (*N*-[4-(4-chloro-phenyl)-1,2,5-oxadiazol-3-yl]-4-trifluoromethyl-benzamide **1**, Figure 1) showed a good antiproliferative activity profile against a panel of 58 tumor cell lines derived from 9 cancer cell types

(GI_{50} values ranging from 6.75×10^{-6} M against renal cancer cell TK10, to 5.46×10^{-7} M, leukemia, HL60TB), and showed Tumor Growth Inhibition (TGI) values lower than 1.00×10^{-4} M in most of the cancer cell lines (3). Therefore, with the aim to integrate the previously obtained data (2), we decided to investigate the effects of phenyl ring substitutions on the antiproliferative activity, by designing (Figure 1) and synthesizing (Figure 2) several compounds related to **1** (2-12), to complete our library (2). Some compounds showed on the phenyl rings the same substituents of **1** but at different positions, while others were substituted with a trifluoromethyl group or with a chlorine atom.

All compounds were tested against HCT116 and HeLa cell lines. Since isomer **4** showed the most interesting growth inhibition results, we also explored its effect on the cellular viability of breast cancer cells (MCF-7 and MDA-MB 468), and the outcomes confirmed the encouraging trend of activity. We also investigated its mechanism of action using as probes its derivatives **20** (Figure 3), bearing fluorescent moieties, and observing their behavior into the cells. Finally, preliminary studies were performed to explore its potential molecular targets.

Materials and Methods

Chemistry. Reagents and solvents were all purchased from Sigma-Aldrich (Saint Louis, MO, USA), except for Oregon Green 488 carboxylic acid succinimide ester obtained from Thermo Fisher Scientific (Waltham, MA, USA), and were used without further purification. Anhydrous solvents were used without further drying. Reactions, performed in conventional conditions or by a microwave synthesizer (Biotage® Initiator; Biotage, Upsala, Sweden) were monitored by thin-layer chromatography on aluminum-backed Silica Gel 60 plates (70-230 mesh; Merck, Darmstadt, Germany), using an ultraviolet fluorescent lamp at 254 nm and 365 nm. Visualization was supported by opportune staining reagents. Purification of intermediates and final compounds was performed *via* flash column chromatography on Geduran Si 60 (40-63 μm, Merck). All the tested compounds were characterized by means of ^{1}H and ^{13}C NMR spectra on a Varian Oxford 300 MHz instrument (Palo Alto, CA, USA), equipped with a non-reverse probe at 25°C, operating at 300 MHz for ^{1}H, 75 MHz for ^{13}C and 282 MHz for ^{19}F. Chemical shifts are expressed as δ (ppm) from tetramethylsilane resonance in the indicated solvent (TMS: δ=0.0

ppm). The coupling constants (*J*-values) are expressed in Hertz (Hz). All spectroscopic data match the assigned structures. Melting points were determined in open capillary tubes on a Buchi Melting Point B-540 (Buchi, Flawil, Switzerland). Mass spectrometry analyses were carried out on an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific) equipped with a Finnigan IonMax Electrospray interface. The purity of the compounds was checked by HPLC analysis (≥95%).

The synthesis of 4-bromo and 4-methyl benzoyl chlorides was performed following the procedure reported in literature (2).

*Synthesis of 1,2,5-oxadiazol-3-amines **17a-g***. The amines **17a-g** were obtained as previously described (Figure 2) (3). The characterization of the new key intermediates **17b** and **17f** is reported below.

*4-(4-methylphenyl)-1,2,5-oxadiazol-3-amine (**17b**)*. Starting compound: 4-methylbenzaldehyde (1.2 mmol). Yield: 8% as white solid (m.p.: 126.7-128.5°C). ^{1}H-NMR (CDCl$_3$): δ 2.38 (s, 3H, CH$_3$), 4.26 (br s, 2H, NH$_2$ exchanged with D$_2$O), 7.36 (d, *J* = 8.2 Hz, 2H, ArH), 7.64 (d, *J* = 8.2 Hz, 2H, ArH) ppm. ^{13}C-NMR (CDCl$_3$): δ 21.44, 122.58, 127.44, 130.09, 140.87, 146.92, 154.31 ppm. ESI-MS: calcd. for C$_9$H$_9$N$_3$O 175, found 176 [M+H]$^+$.

*4-(4-bromophenyl)-1,2,5-oxadiazol-3-amine (**17f**)*. Starting compound: 4-bromobenzaldehyde (2.70 mmol). Yield: 18% as light brown solid (m.p.: 143.8-144.8°C). ^{1}H-NMR (CDCl$_3$): δ 4.20 (br s, 2H, NH$_2$ exchanged with D$_2$O), 7.62 (d, *J* = 8.3 Hz, 2H, ArH), 7.68 (d, *J* = 8.3 Hz, 2H, ArH) ppm. ^{13}C-NMR (CDCl$_3$): δ 125.02, 125.89, 129.38, 135.68, 146.70, 154.56 ppm. ESI-MS: calcd. for C$_8$H$_6$BrN$_3$O 240, found 241 [M+H]$^+$.

*Synthesis of 1,2,5-oxadiazol-3-amides **1-12** and **18***. Compounds **1-4**, **7-9** and **12** were obtained as previously reported (2), while the new derivatives **5**, **6**, **10,11** and **18** were synthesized using the following the procedures.

*Synthesis of 4-bromo-N-(4-(4-chlorophenyl)-1,2,5-oxadiazol-3-yl)benzamide (**5**)*. 4-Bromobenzoyl chloride (0.306 mmol) was added to a solution of **17c** (0.255 mmol) and 4-dimethylaminopyridine (0.306 mmol) in dry 1,2-dichloroethane (1.5 ml), at room temperature, under nitrogen atmosphere. The reaction mixture was irradiated in a microwave synthesizer at 120°C and 300 W for 45 min. The solution was treated with water (1.5 ml) and extracted with dichloromethane (3 × 1 ml). The collected organic

phases were washed with 1N HCl, dried over Na_2SO_4, filtered and evaporated under reduced pressure to give a residue, which was purified by flash column chromatography (eluent mixture: cyclohexane/ethyl acetate, 80:20).

Yield: 11% as brown solid (m.p.: 195.5-197.0°C). ^{1}H-NMR ($CDCl_3$): δ 7.47 (d, J=8.1 Hz, 2H, ArH), 7.65 (d, J=8.7 Hz, 2H, ArH), 7.68 (d, J=8.7 Hz, 2H, ArH), 7.76 (d, J=8.1 Hz, 2H, ArH), 8.18 (br s, 1H, NH exchanged with D_2O) ppm. ^{13}C-NMR ($CDCl_3$): δ 123.78, 127.46, 129.16, 129.25, 129.56, 131.80, 133.18, 135.03, 147.18, 153.76, 166.48 ppm. ESI-MS: calcd. for $C_{15}H_9BrClN_3O_2$ 378, found 377 [M-H]$^-$.

General procedure for the synthesis of amides 6, 10, 11 and 18. To a suspension of NaH 60% dispersion in mineral oil (0.3 mmol) in dry *N-N*-dimethylformamide (3 ml) cooled on an ice bath under nitrogen atmosphere, the appropriate 4-substituted phenyl-1,2,5-oxadiazol-3-amine (0.25 mmol) was added, and the mixture was stirred at 0°C for 20 min. Subsequently, the suitable acyl chloride (0.3 mmol) was added dropwise at 0°C and the mixture was stirred at 60°C for 12 h. After the addition of water (3 ml), *N-N*-dimethylformamide was removed under reduced pressure and the obtained residue was extracted with ethyl acetate (3× 2 ml). The organic phase was dried over Na_2SO_4 and evaporated under *vacuum* to obtain the crude product, which was purified by flash column chromatography (eluent mixture: cyclohexane/ethyl acetate, 80:20).

4-methyl-N-(4-(4-chlorophenyl)-1,2,5-oxadiazol-3-yl)benzamide (6). Starting compounds: **17c** and 4-methylbenzoyl chloride. Yield: 24% as brown solid (m.p.: 163.5-164.0°C). ^{1}H-NMR ($CDCl_3$): δ 2.37 (s, 3H, CH_3), 7.25 (d, J=8.4 Hz, 2H, ArH), 7.38 (d, J=8.4 Hz, 2H, ArH), 7.58 (d, J=8.6 Hz, 2H, ArH), 7.70 (d, J=8.6 Hz, 2H, ArH), 8.07 (br s, 1H, NH exchanged with D_2O) ppm. ^{13}C-NMR ($CDCl_3$): δ 21.91, 124.31, 127.89, 129.06, 129.17, 129.79, 130.06, 137.27, 144.59, 149.13, 149.75, 165.50 ppm. ESI-MS: calcd. for $C_{16}H_{12}ClN_3O_2$ 313, found 312 [M-H]$^-$.

4-trifluoromethyl-N-(4-(4-bromophenyl)-1,2,5-oxadiazol-3-yl)benzamide (10). Starting compounds: **17f** and 4-trifluoromethylbenzoyl chloride. Yield: 20% as off-white solid (m.p.: 242.5-244.8°C). ^{1}H-NMR ($CDCl_3$): δ 7.57 (d, J=8.7 Hz, 2H, ArH), 7.64 (d, J=8.7 Hz, 2H, ArH), 7.81 (d, J=8.2 Hz, 2H, ArH), 8.00 (d, J=8.2 Hz, 2H, ArH), 8.15 (br s, 1H, NH exchanged with D_2O) ppm. ^{13}C-NMR ($CDCl_3$):

δ 121.50, 122.76, 125.12, 126.33, 129.22, 129.25, 133.18, 135.19, 135.42, 135.63, 151.41, 170.08 ppm. ^{19}F-NMR (CD$_3$OD): δ -63.63 (s, CF$_3$) ppm. ESI-MS: calcd. for C$_{16}$H$_9$BrF$_3$N$_3$O$_2$ 412, found 411 [M-H]$^-$

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4-trifluoromethyl-N-(4-(4-methylphenyl)-1,2,5-oxadiazol-3-yl)benzamide (11). Starting compounds: **17b** and 4-trifluoromethylbenzoyl chloride. Yield: 19% as gray solid (m.p.: 118.8-121.5°C). ^{1}H-NMR (CDCl$_3$): δ 2.44 (s, 3H, CH$_3$), 7.36 (d, *J*=8.2 Hz, 2H, ArH), 7.57 (d, *J*=8.2 Hz, 2H, ArH), 7.79 (d, *J*=8.6 Hz, 2H, ArH), 7.98 (d, *J*=8.6 Hz, 2H, ArH), 8.12 (br s, 1H, NH exchanged with D$_2$O) ppm. ^{13}C-NMR (CDCl$_3$): δ 31.15, 122.31, 125.69, 125.78, 126.45 (q), 126.25, 127.69, 127.89, 128.32, 130.29, 130.45, 134.31, 141.66, 148.57, 150.20, 165.78 ppm. ^{19}F-NMR (CD$_3$OD): δ -63.75 (s, CF$_3$) ppm. ESI-MS: calcd. for C$_{17}$H$_{12}$F$_3$N$_3$O$_2$ 347, found 346 [M-H]$^-$.

4-chloro-2-nitro-N-(4-(4-(trifluoromethyl)phenyl)-1,2,5-oxadiazol-3-yl)benzamide (18). Starting compounds: **17g** and 4-chloro-2-nitrobenzoyl chloride. Yield: 52% as yellowish solid (m.p.: 208-209°C). ^{1}H-NMR (CD$_3$OD): δ 7.77 (d, *J*=8.2 Hz, 1H, ArH), 7.86 (d, *J*=5.3 Hz, 2H, ArH), 7.90 (d, *J*=2.0 Hz, 1H, ArH), 8.00 (d, *J*=8.4 Hz, 2H, ArH), 8.22 (d, *J*=2.0 Hz, 1H, ArH) ppm. ^{13}C-NMR (CD$_3$OD): δ 118.60-122.18-125.67-129.40 (q), 123.02, 125.96-126.02-126.08-126.16 (q), 127.59, 129.98, 130.42, 130.99, 131.54, 132.78-133.21-133.67-134.05 (q), 133.52, 146.98, 147.37, 153.30, 165.07 ppm. ^{19}F-NMR (CD$_3$OD): -63.04 ppm. ESI-MS: calcd. for C$_{16}$H$_8$ClF$_3$N$_4$O$_4$ 412, found 411 [M-H]$^-$.

Synthesis of 2-amino-4-chloro-N-(4-(4-(trifluoromethyl)phenyl)-1,2,5-oxadiazol-3-yl)benzamide (19)

To a solution of benzamide **18** (0.242 mmol) in ethyl acetate (15.61 ml), SnCl$_2$ (1.212 mmol) was added and the mixture was refluxed for 3 h. A saturated aqueous solution of NaHCO$_3$ was added until pH 7-8 and the precipitated tin salts were filtered. The aqueous phase was extracted with ethyl acetate (3 × 3 ml). The collected organic layers were dried over Na$_2$SO$_4$, filtered and evaporated under *vacuum*. The obtained residue was purified by flash column chromatography (eluent mixture: cyclohexane/AcOEt, 8:2) to afford intermediate **19**.

Yield: 79% as white solid (m.p.: 155-156°C). ^{1}H-NMR (CD$_3$OD): δ 6.63 (dd, *J*=8.6, 2.1 Hz, 1H, ArH), 6.78 (d, *J*=2.1 Hz, 1H, ArH), 7.71 (d, *J*=8.6 Hz, 1H, ArH), 7.79 (d, *J*=8.4 Hz, 1H, ArH), 7.94 (d, *J*=8.4 Hz, 1H, ArH) ppm. ^{13}C-NMR (CD$_3$OD): δ 115.02, 115.49, 117.50, 118.57-122.16-125.70-129.30 (q), 125.96-126.04-126.11-126.16 (q), 127.60, 130.01, 130.47, 132.78-133.24-133.68-134.10

(q), 138.07, 146.40, 150.25, 153.31,165.59 ppm. ^{19}F-NMR (CD$_3$OD): δ -63.07 ppm. ESI-MS: calcd. for C$_{16}$H$_{10}$ClF$_3$N$_4$O$_2$ 382, found 383 [M+H]$^+$.

Synthesis of N-{5-chloro-2-[4-(4-trifluoromethyl-phenyl)-1,2,5-oxadiazol-3-ylcarbamoyl]-phenyl}-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-terephthalamic acid and N-{5-chloro-2-[4-(4-trifluoromethyl-phenyl)-1,2,5-oxadiazol-3-ylcarbamoyl]-phenyl}-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-iso

phthalamic acid mixture (20). Oregon Green 488 carboxylic acid succinimide ester (0.078 mmol) was added to a solution of amine **19** (0.078 mmol) and *N-N*-diisopropylethylamine (0.39 mmol) in dry *N-N*-dimethylformamide (12 ml), and the mixture was stirred for 5 h at room temperature (12). After the removal of dimethylformamide under *vacuum*, the residue was initially filtered on silica and then purified by flash column chromatography (eluent mixture: dichloromethane/methanol, 90:10) providing the mixture **20**. Yield: 23% as yellow oil. ESI-MS: calcd. for C$_{37}$H$_{20}$ClF$_3$N$_4$O$_8$ 741, found 763 [M+Na]$^+$.

Antiproliferative assay on HCT-116 cell line.

Cell line culture. The human colorectal cancer cell line HCT-116 (Cell-Lab Unimore, University of Modena and Reggio Emilia, Italy) was cultured in Dulbecco's modified Eagle medium (DMEM; Euroclone, Devon, UK), supplemented with 10% heat-inactivated fetal bovine serum (Euroclone) and 1% Pen/Strep (Euroclone). Cultures were equilibrated with humidified 5% carbon dioxide in air at 37°C. All studies were performed in *Mycoplasma*-negative cells, as routinely determined with the MycoAlert Mycoplasma detection kit (Lonza, Walkersville, MD, USA) (2).

Cytotoxicity screening. The cytotoxicity of the compounds was determined by MTT assay (13,14). Briefly, the cells were seeded into 96-well plates and cultured overnight. Various concentrations of the test compounds dissolved in DMSO were then added and incubated for 48 h. After incubation, 10% v/v of a solution of 0.5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma-Aldrich) was added and then incubated at 37°C for 3 h. The medium was removed and 100 μl DMSO were added to dissolve the dark blue crystals. After incubation for 30 min at room temperature to ensure the dissolution of all crystals, absorbance was measured using an ELISA plate reader (TECAN, Männedorf, Switzerland) at 595 nm (2).

Antiproliferative assay on HeLa cell line

Cell line culture. The human cervix adenocarcinoma cell line HeLa was grown in Nutrient Mixture F-12 [HAM] (Sigma Chemical Co., St. Louis, MO USA) supplemented with 10% Heat-inactivated fetal calf serum (FCS; Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B (Sigma Chemical Co., St. Louis, MO USA). The cells were cultured at 37°C in a moist atmosphere of 5% carbon dioxide in air (11).

Cytotoxicity screening. HeLa ($4\text{-}5 \times 10^4$) cells were seeded into a 24-well cell culture plate. After incubation for 24 h, various concentrations of the test agents were added, and the cells were further incubated in standard conditions for 48 h. A trypan blue assay (15) was performed to determine cell viability. Cytotoxicity data were expressed as GI_{50} values, i.e. the concentration of the test agent inducing a 50% reduction in the cell count, compared to control cultures (11).

Cellular viability of MCF-7 and MDA-MB 468 cell lines.

Cell line culture. Two breast cancer cell lines were used, namely MCF-7 (ATCC: HTB-22) and MDA-MB 468 (ATCC: HTB-132). Both cell lines were cultured in DMEM supplemented with 10% FCS and 1% GlutaMAX (BioConcept, Allschwil, Switzerland). Cells were kept at 37°C in a humidified atmosphere supplemented with 5% carbon dioxide.

Cell viability. Cell viability was determined through the assessment of the metabolic activity. Briefly, MCF-7 (ATCC: HTB-22) or MDA-MB 468 (ATCC: HTB-132) cells were seeded at a density of 5,000 cells/well in 96-well plates (Eppendorf, Schönenbuch, Switzerland). Compound **4** was solubilized in DMSO at a final concentration of 15 mM and stored at 4˚C until further use. One day after the seeding, the cells were exposed to a medium containing a serial dilution of compound **4**. After a 48-h incubation, cell viability was determined using the resazurin viability fluorometric cell viability kit (PromoCell GmbH, Heidelberg, Germany) according to the manufacturer's instruction. Fluorescence was detected using the TECAN Infinite M200Pro plate reader (Ex=530 nm, Em=590 nm). Viability was calculated as fold of solvent control, after subtracting the fluorescence of the medium. Data were plotted using

Graph Pad Prism (version 6.07, La Jolla, CA, USA); the inhibitory potency was estimated calculating the GI_{50} value using a nonlinear fit regression.

MeT-5A inhibition growth assay. MeT-5A (human mesothelium) cells were grown in RPMI-1640 (Sigma Chemical Co.), supplemented with 2.4 g/l Hepes, 0.11 g/l Na-pyruvate, 2.5 g/l glucose, 2g/l Na-bicarbonate. Heat-inactivated FCS 10%, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B (Sigma Chemical Co.) were added to the medium. The cells were cultured at 37 °C in a moist atmosphere of 5% carbon dioxide in air. Cells (3×10^4) were seeded into a 24-well cell culture plate. After incubation for 24 h, various concentrations of the test agents were added to the complete medium and the cells were further incubated for 48 h. A trypan blue assay was performed to determine the cell viability. Cytotoxicity data were expressed as GI_{50} values, i.e., the concentration of the test agent inducing 50% reduction in the number of cancer cells, compared to control cultures.

Microscope fluorescence analysis. The breast cancer cell line MCF-7 (ATCC HBT-22) was used for the assessment of microtubule interaction with the fluorescent mixture **20**. MCF-7 cells were cultured in supplemented DMEM on glass cover slips until the 70% confluence was reached. Then, the fluorescent mixture **20** was added to the cell culture at a concentration of 1, 10, and 100 µM and incubated for 24 h at 37°C, 5% carbon dioxide in humidified atmosphere.

Fluorescence microscopy. Fluorescence microscopy was performed using an inverted fluorescent microscope Leica DM IL LED (Heerbrugg, Switzerland). Briefly, MCF-7 cells were fixed in paraformaldehyde 4% for 15 min at room temperature. After three washes in phosphate buffer saline (PBS) at pH 7.4, cells were stained first with phalloidin 555 (Thermo Fischer, Zug, Switzerland) for 20 min and then with Hoechst 33342 (Thermo Fisher) for 10 min. All cells were co-stained with phalloidin 555 (staining F-actin of the cytoskeleton) and Hoechst 33342 (staining DNA). Three final washes were then performed before mounting the glass slides, and then fluorescent microscopy was performed. Images were taken using the software Leica suite X (LASX), and they were analyzed using the Image Processing Software.

Topoisomerase-mediated DNA relaxation assay. Supercoiled pBR322 plasmid DNA (0.25 µg; Fermentas Life Sciences, Burlington, Ontario, Canada) was incubated with 1 U topoisomerase II (human recombinant topoisomerase II α; USB Corporation, Cleveland, OH, USA) and the test compounds as indicated, for 60 min at 37°C in 20 µl reaction buffer. Reactions were stopped by adding 4 µl stop buffer (5% sodium dodecyl sulfate (SDS), 0.125% bromophenol blue, and 25% glycerol), 50 µg/ml proteinase K (Sigma-Aldrich) and were further incubated for 30 min at 37°C. The samples were separated by electrophoresis on a 1% agarose gel at room temperature. The gels were stained with ethidium bromide 1 µg /ml in TAE buffer (0.04 M Tris-acetate and 0.001 M EDTA) and transilluminated by UV light; fluorescence emission was visualized by a CCD camera, coupled to a Bio-Rad Gel Doc XR apparatus (11).

Statistical analysis. For statistical analysis, the software GraphPad Prim was used. For statistical analysis of the cell viability the One-way ANOVA with Dunett's multi comparison test was used. The two-way ANOVA was applied for statistical analysis of the cell counting, while two data points comparing the GI_{50}-values was analyzed using an unpaired t-test. A p-value <0.05 was considered statistically significant.

Results and Discussion

Chemistry. Our library of 1,2,5-oxadiazole derivatives, including the previously reported products **1-4**, **7-9**, **12,** was integrated with the new analogues **5**, **6**, **10**, **11**, obtained following the synthetic pathway described in the literature, and depicted in Figure 2 (2). Compound **20** was afforded as a mixture of regioisomers, as described in Figure 3.

The suitable commercially available benzaldehydes were treated with hydroxylamine to give oximes **13a-g**, which were then converted into the corresponding chloro-oximes **14a-g** in the presence of *N*-chlorosuccinimide**.** The chlorine atom was substituted with a cyano group by treatment with potassium cyanide (**15a-g**). Intermediates **15a-g** were reacted in the presence of hydroxylamine to

afford the corresponding amino-bis-oximes (acetamidines) **16a-g** that were cyclized in basic condition to the amines **17a-g** (16). The latter were coupled with the suitable benzoyl chlorides in the presence of sodium hydride to afford the products **1-12** and the amide **18** (Figure 2) (2). Finally, the nitro group of intermediate **18** was reduced by treatment with tin chloride to afford the amine **19**, which was coupled with Oregon

Antiproliferative activity on HCT-116 and HeLa cell lines. To complete our previous set of data (2), compounds **5**, **6**, **10** and **11** were tested on HCT-116 cell line (human colorectal cancer); then, to widen the antiproliferative profile of this class, all the derivatives were also assayed on HeLa cells (human cervix adenocarcinoma). The results are reported in Table I.

The new compounds exhibited a lower antiproliferative activity against HCT-116 cell line in comparison with the previously synthesized derivatives (11); similarly, the GI_{50} values on HeLa cells were generally higher with respect to the majority of those obtained for the previous candidates. Among this set, compound **4** showed the highest activity *versus* HeLa cell line, confirming its promising antiproliferative effect, previously observed on HCT-116 cells (GI_{50}=1.48 μM). Therefore, it was also tested against the breast cancer cell lines MCF-7 and MDA-MB 468.

Cellular viability of MCF-7 and MDA-MB 468 cell lines. The effect of compound **4** on the viability of MCF7 and MDA-MB 468 cells was assessed by measuring their metabolic activity, through the resazurin fluorometric cell viability kit. The results of the test are reported in Figure 4**.**

The assay revealed a significant inhibition of metabolic activity upon treatment with compound **4**, resulting in a reduction of resazurin to resafurin. In both cell lines, compound **4** exhibited a comparable potency with an GI_{50} of 8.50 μM in MCF-7 and an GI_{50} of 10.59 μM in MDA-MB 468 cells (Student's t-test, $p>0.05$, Figure 4).

Antiproliferative activity on MeT-5A. Compound **4**, endowed with the highest antiproliferative activity, and compound **1**, as reference, were tested on MeT-5A cell line, showing GI_{50} values of 7.3 μM and 9.3 μM respectively, after 48 h of incubation. The compounds exhibited a lower cytotoxicity against healthy cells with respect to the tested cancer cell lines, suggesting a certain degree of specificity.

Fluorescent microscopy analysis. Following the incubation of MCF-7 breast cancer cells with the fluorescent mixture **20**, a lower viability was observed compared to the control group, in which no compound was added. Three different concentrations (1, 10 and 100 μM) of the compound were used, but only the highest gave a relevant fluorescent signal (Figure 5).

The fluorescence microscopy analysis suggested an overlay between the actin and the fluorescent derivative of compound **4** (mixture **20**), without showing substantial alterations of the cellular shape. Therefore, the affinity of the molecule towards the actin filament indicates a potential interaction may occur.

AlphaScreen-based assay. Several literature works have reported that oxadiazole-based derivatives can inhibit the signal transducer and activator of transcription 3 (STAT3) transcription factor, by binding to its SH2 domain (10, 17–19). Therefore, the potential inhibitory activity of the most interesting compounds (**1**,**2** and **4**) was tested at 30 μM concentration by an AlphaScreen-based assay, though without positive results (data not shown).

Topoisomerase-mediated DNA relaxation assays. Some oxadiazole derivatives have also been reported to have inhibitory activity on topoisomerases (20, 21), so the same three compounds, endowed with the highest antiproliferative activity (**1**,**2** and **4**), were tested against topoisomerase II (Table II).

All the tested compounds showed an interesting inhibitory effect on topoisomerase II relaxation activity: the results of the assay revealed a complete enzyme inhibition at a concentration of 100 μM.

Conclusions

To gain new insights into the effect of phenyl substitution on the antiproliferative properties of the previously reported compound **1**, a small set of oxadiazoles was synthesized and tested on different human tumor cell lines. Among the synthesized derivatives, compounds **2** and **4** showed the most interesting antiproliferative activity, highlighting that only slight modifications on compound **1**, such as the shift of the CF_3 group to the *meta* position (**2**) or the inversion of the position of the substituents (**4**), are well tolerated. Interestingly, the antiproliferative effect of **4** on non-tumorigenic MeT-5A cells

appeared lower with respect to that exerted on tumor cells, suggesting a certain specificity. Further preliminary studies suggested that the antiproliferative properties of this compound could be related to the interaction with microtubules and to the ability to inhibit the relaxation activity of topoisomerase II. Therefore, these results confirm that compound **4** could be considered as a promising antiproliferative agent, endowed with a good specificity for the tested cancer cell lines.

Acknowledgements

Authors' Contributions

DE and FP performed cytotoxicity assays and HE stainings of MCF-7 and MDA-MB-468 cells.

HEM financed the studies. DE, FP, HEM wrote the respective parts of the manuscript.

LDV and ANGA performed biological assay on HeLa, MeT-5A and human topoisomerase II α.

LB supported the design of the compounds.

MM, FM, AG and SV designed, synthesized and planned all the experiments and wrote the manuscript.

SV financed the studies.

Conflict of interest

The authors declare no competing interests.

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Figure 1
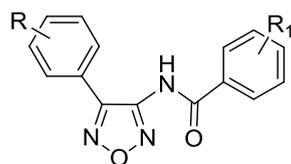
1-12

R, R$_1$= H, CH$_3$, CF$_3$, Cl, Br

Compound	R	R$_1$	Compound	R	R$_1$
1	*p*-Cl	*p*-CF$_3$	**7**	*p*-Cl	H
2	*p*-Cl	*m*-CF$_3$	**8**	*m*-Cl	*p*-CF$_3$
3	*p*-Cl	*o*-CF$_3$	**9**	*o*-Cl	*p*-CF$_3$
4	*p*-CF$_3$	*p*-Cl	**10**	*p*-Br	*p*-CF$_3$
5	*p*-Cl	*p*-Br	**11**	*p*-CH$_3$	*p*-CF$_3$
6	*p*-Cl	*p*-CH$_3$	**12**	H	*p*-CF$_3$

Figure 2

R	R_1 = H, p-CH$_3$, p-Cl, p-Br, p-CF$_3$, m-CF$_3$, o-CF$_3$
a = H	
b = p-CH$_3$	
c = p-Cl	
d = m-Cl	
e = o-Cl	
f = p-Br	
g = p-CF$_3$	
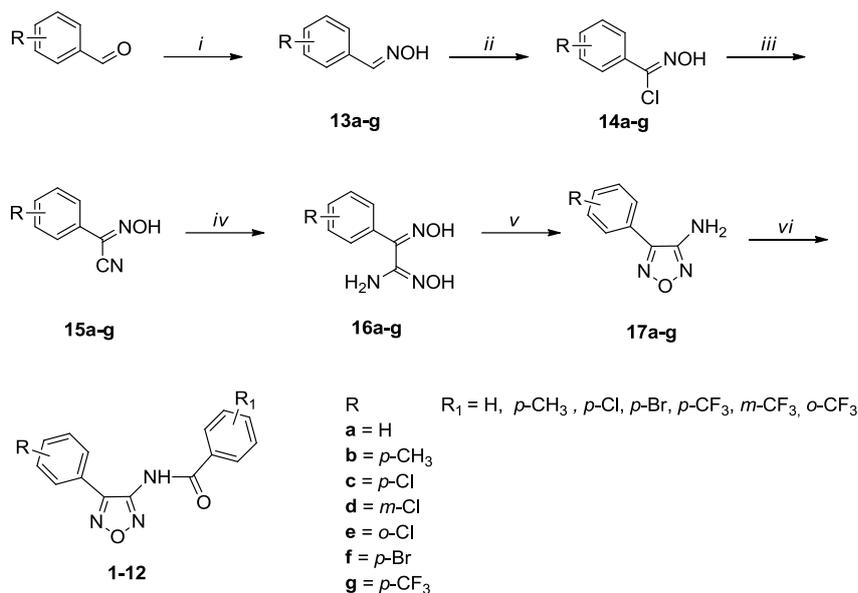
Reagents and conditions: (*i*) NH$_2$OH HCl, NaHCO$_3$, methanol, reflux, 2 h; (*ii*) NCS, *N,N*-dimethylformamide, rt, 12 h; (*iii*) KCN, diethyl ether/H$_2$O, rt, 5 h; (*iv*) NH$_2$OH HCl, NaHCO$_3$, methanol, reflux, 12 h; (*v*) 2N NaOH, reflux, 12 h; (*vi*) for **5**: 4-bromobenzylchloride, 4-dimethylaminopyridine, 1,2-dichloroethane, mw, 120°C, 45 min; for **6**,**10** and **11**: R$_1$COCl, 60% NaH, dry *N,N*-dimethylformamide, N$_2$, 60°C, 12 h.

Figure 3

Reagents and conditions: (*i*) a. 60% NaH, dry *N,N*-dimethylformamide, N$_2$, 0°C, 30 min.; b. 4-chloro-2-nitrobenzoyl chloride, 60°C, 12 h; (*ii*) SnCl$_2$, ethylacetate, reflux, 3 h; (*iii*) 5(6)-carboxyfluoresceine, *N,N*-diisopropylethylamine, dry *N,N*-dimethylformamide, N$_2$, r.t., 5 h.
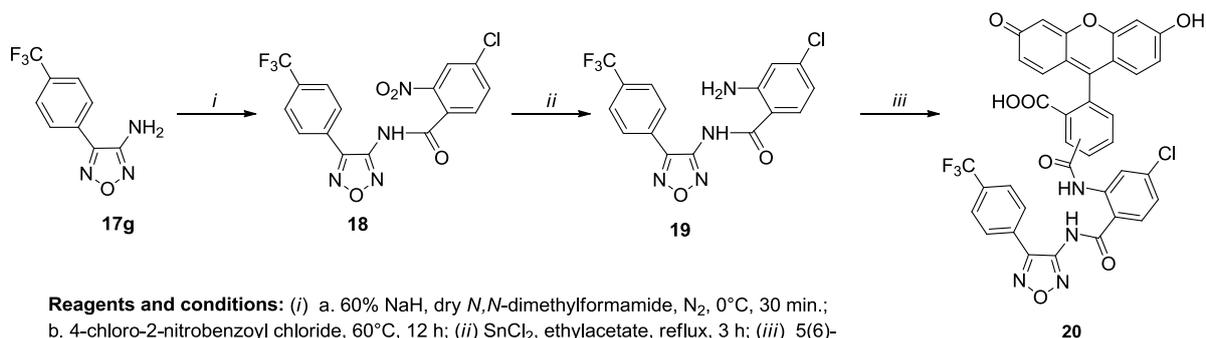
Table I. *Antiproliferative activity of compounds 1-12 against the human colorectal cancer (HCT-116) and cervix adenocarcinoma (HeLa) cell lines, evaluated after 48 hours of exposure to the compounds (2, 11).*
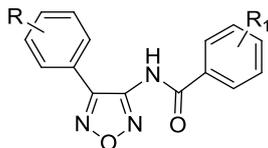
Entry	R	R_1	HCT-116 GI_{50} μM	HeLa GI_{50} μM	Entry	R	R_1	HCT-116 GI_{50} μM	HeLa GI_{50} μM
1	*p*-Cl	*p*-CF$_3$	1.3±0.2	3.6±0.4	7	*p*-Cl	H	n.a.	54.5±12.7
2	*p*-Cl	*m*-CF$_3$	7.4±1.15	4.1±0.8	8	*m*-Cl	*p*-CF$_3$	15.4±2.8	5.7±1.1
3	*p*-Cl	*o*-CF$_3$	n.a.	74.5±10.2	9	*o*-Cl	*p*-CF$_3$	56.7±3.1	19.2±2.3
4	*p*-CF$_3$	*p*-Cl	0.95±0.02	1.48±0.3	10	*p*-Br	*p*-CF$_3$	47.7±6.3	29.2±4.2
5	*p*-Cl	*p*-Br	85.6±16	18.6±3.5	11	*p*-CH$_3$	*p*-CF$_3$	56.5±9.1	36.0±5.4
6	*p*-Cl	*p*-CH$_3$	47.2±5.4	23.2±1.8	12	H	*p*-CF$_3$	n.a.	27.0±0.4

GI_{50} values (μM) are means±SD of at least three separate experiments; n.a.: not active (≥ 100).
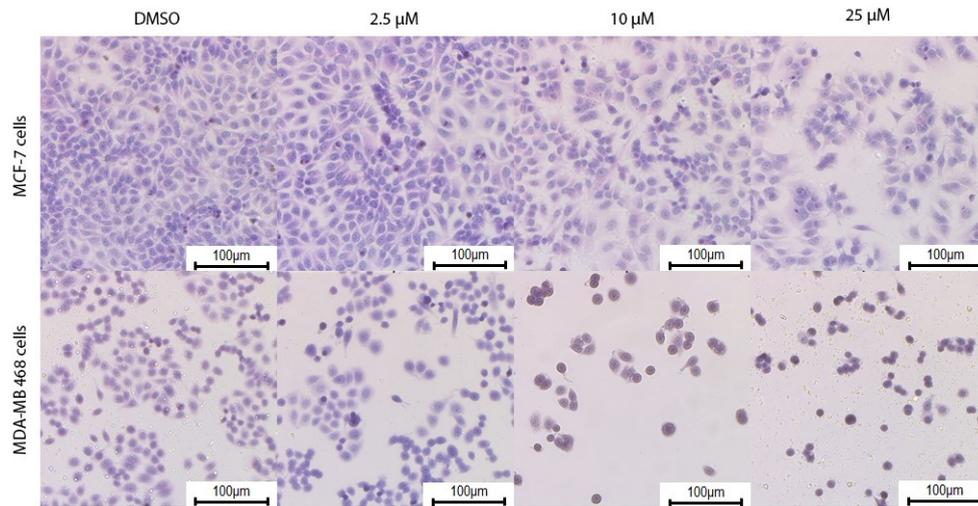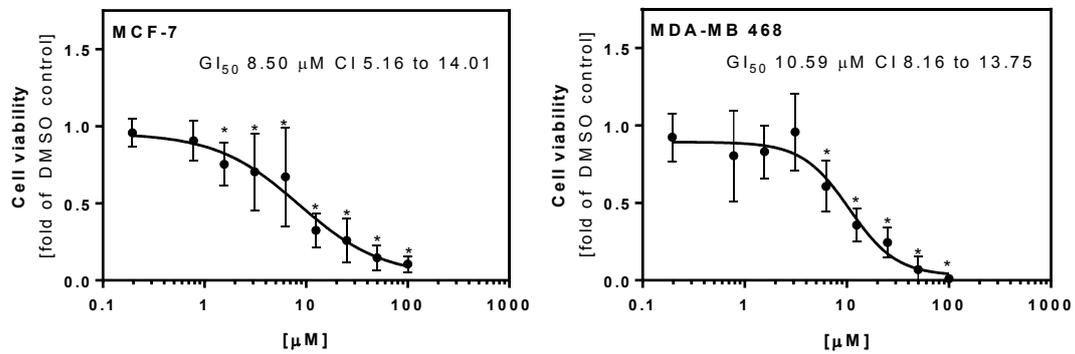
Figure 4. *Effect of compound **4** on the viability of breast cancer cells (at the top); specifically, on the ER-positive MCF-7 (A) and the ER-negative MDA-MB 468 (B) cell lines. The viability of cells exposed to serial dilutions of compound **4** for 48 h was assessed by quantifying the enzymatic reduction of resazurin. The experiments (n=4) were conducted in triplicates. Data are presented as mean ± SD. * p<0.05 One-way ANOVA compared to control.*

A

B

C
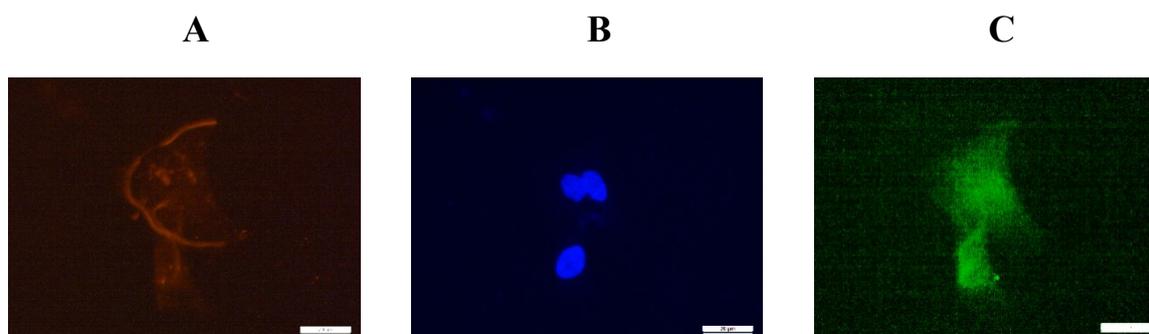
Figure 5. *Images showing the presence of the fluorescent compound **20** in 488 MCF-7 breast cancer cells. Phalloidin Alexa Fluor 555 dye staining (A); Hoechst 3342 staining (B); Fluorescent mixture **20** staining (C).*

Table II*. Effect of compounds **1**,**2** and **4** on the relaxation of supercoiled plasmid DNA by topoisomerase II (TopoII).*
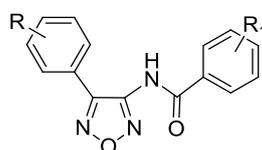
Compound	R	R_1	Topo II inhibitory effect (100 µM)
1	p-Cl	p-CF$_3$	+++
2	p-Cl	m-CF$_3$	+++
4	p-CF$_3$	p-Cl	+++

+++ complete inhibition; ++- partial inhibition, +-- weak inhibition, --- no inhibition