Exploration of novel heterofused 1,2,4-triazine derivatives in colorectal cancer

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Abstract

Experimental Approach: Colorectal cancer (CRC) is the third leading cause of cancer-related deaths in men and in women. It's expected to cause about 53,200 deaths during 2020. An effective drug for therapy and prognosis after surgery still does not exist. Therefore, the search for new lead structures and chemical entities for the development of new effective anticancer agents is an increasingly important task in medicinal chemistry. This trend of global research includes work on the use of 1,2,4-triazine scaffold as a source for the design of biologically relevant molecules with well-known broad biological applications. A series of new pyrazolo[4,3-e]tetrazolo[4,5-b][1,2,4]triazine sulfonamides were designed, synthesized, and assessed as anticancer activity agents. Experimental Approach: The impact of two selected compounds, MM-128, and MM-129 (MMs), were evaluated against human colon cancer in in vitro and in zebrafish embryo xenograft model. Key Results: Our results show that the new synthesized compounds effectively inhibit cell survival and DNA synthesis in both DLD-1 and HT-29 cell lines. Their effectiveness is much higher as compared with the standard chemotherapy used for colorectal cancer, i.e. 5-fluorouracil. Flow cytometry analysis after annexin V-FITC and propidium iodide staining revealed that apoptosis was the main response of colorectal cancer cells to MMs treatment. We also found that MM-129 effectively inhibits tumor development in both DLD-1 and HT-29 zebrafish xenografts. Conclusion and Implications: New pyrazolo[4,3-e]tetrazolo[4,5-b][1,2,4]triazine sulfonamides may be new candidates for further evaluation as chemotherapeutic agents against colorectal cancer.

Abbreviations

5-FU: 5-fluorouracil **AIF** : apoptosis inducing factor**Apaf-1:** apoptotic protease activating factor 1 **Bak:**Bcl-2 - homologous antagonist/killer **Bax:** Bcl-2-associated X protein **Bcl-2:** antiapoptotic protein **Bcr-Abl:** tyrosine kinase **Bid:** Bax-like BH3 protein **tBid:** truncated BID**DLD-1:** cell line of human colorectal adenocarcinoma**FADD:** Fas-associated death domain protein **HT-29:** cell line of human colorectal adenocarcinoma **hpf:** hours post-fertilization **hpt:** hour post-treatment **MMP:**mitochondrial membrane potential **MMs:** MM-128, MM-129**PCD** : programmed cell death **PS:** phosphatidyloserine**ROS** : roscovitine

INTRODUCTION

The 1,2,4-triazine ring is an eminent structural motif found in plentiful natural and synthetic biologically active compounds (Cascioferro, Parrino, et al., 2017; Yurttas, Ciftci, et. al., 2017). Among the known various biological activities of 1,2,4-triazines and their related benzo- and heterofused derivatives, antitumor activity deserves special attention. Recently, a few reviews on the chemistry and the biological properties of this class of compounds have been published (Kumar, Sirohi, et al., 2014; Rao, Kumar, et al., 2016; Arshad, Khan, et al., 2014).

It should be emphasized that compounds with the 1,2,4-triazine nucleus condensed with five-membered heterocycles have received considerable attention because they are bioisosteric with a purine core. Among the fused 1,2,4-triazine derivatives with one heterocycle, compounds bearing a pyrrole ring, such as pyrrolo[2,1-*c*][1,2,4]triazine and pyrrolo[2,1-*f*][1,2,4]triazine, represent the most abundant class of triazine with antitumor activity. Derivatives bearing this heterocyclic system are widely described as potent kinase inhibitors. Pyrrolo[2,1-*f*][1,2,4] triazine scaffold, miming the adenine ring of ATP, was also employed to afford other kinase inhibitors such as Met kinase inhibitors (Borzilleri, Chen, et al., 2007).

Another interesting and little studied in the group of 1,2,4-triazines condensed with a five-membered heterocycle is the pyrazolo[4,3-*e*][1,2,4]triazine ring system. Its derivatives are the least known in the group of condensed pyrazolotriazines and were less studied compared to pyrrolotriazines. Some synthesized derivatives of this system were evaluated for their anticancer activity against five type tumor cell lines (CEM, CEM-DNR, K-562, K-562-tax, and A549) and they showed antiproliferative activity against A549 cell line in the micromolar range (Gucký, Frysova, et al., 2009; Gucký, Reznícková, et al., 2010). Another group is pyrazolo[4,3-*e*][1,2,4]triazine sulfonamides prepared as inhibitors of carbonic anhydrase hCA IX and XII with antitumor activity (Mojzych, Bielawska, et al., 2014; Mojzych, Ceruso, et al., 2015). Moreover, some previously described pyrazolotriazine sulfonamides were found to have dose-dependent antiproliferative effects against two Bcr-Abl positive cancer cell lines, K562 and BV173. Therefore, they were tested against Abl kinase (Mojzych, Subertova, et al., 2014).

On the other hand, our previous study showed that tricyclic derivatives of the pyrazolo[4,3-*e*][1,2,4]triazines condensed with triazole or tetrazole were the most active against cancer cell lines (Mojzych, 2011; Mojzych, Tarasiuk, et. al. 2018). Replacement of triazole with a tetrazole ring results in decrease of the anticancer activity.

Based on the literature above, we designed a novel series of pyrazolo[4,3-*e*]tetrazolo[4,5-*b*][1,2,4]triazine sulfonamides differing in the structure of the sulfonamide group. They are a unique group of 22 (MM-118 - MM-139) compounds that effectively inhibit DNA synthesis and cell survival of four types of cancer: colorectal cancer, breast cancer, gastric cancer, and leukemia. Screening results revealed that compounds MM-128 and MM-129 (MMs) presented in Figure 1 exhibited strong inhibition activity toward DLD-1 and HT-29 (two of the cell lines of CRC). They showed potent antiproliferative effects against colon cancer cell lines with IC_{50} values ranging from 1.1 to 3.1 μM compared to 5-fluorouracil (5-FU) and roscovitine (ROS) with values above 10 μM. MMs have a similar chemical structure to ROS, which is in the clinical trial phase. We also evaluated the mechanism of action of two novel sulfonamide derivatives and found that they play anticancer roles through the activation of apoptosis. In this study, we successfully developed zebrafish xenografts with human DLD-1 and HT-29 colorectal cancer cells and validated these models with anticancer drug 5-FU, used clinically to treat cancer patients. We found that MM-129 compound with the highest pharmacological activity effectively inhibits tumor development in both DLD-1 and HT-29 xenografts.

METHODS

Synthesis

General

Melting points were determined on a Mel-Temp apparatus and were uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Varian spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C). The chemical shift values were expressed in ppm (part per million) with tetramethylsilane (TMS) as an internal reference. The relative integrals of peak areas agreed with those expected for the assigned structures. The molecular weight of the final compounds was assessed by electrospray ionization mass spectrometry (ESI/MS) on Agilent Technologies 6538 UHD Accurate Mass Q-TOF LC/ MS (Lublin, Poland). Elemental compositions were within ±0.4% of the calculated values. For preparation and spectroscopic data of compounds **1-2,** see the literature (Mojzych & Rykowski, 2004).

General method synthesis of derivative (3) (Scheme 1)

Compound **2** (1.156 g, 4 mmol) was added portion wise to stirred and cooled chlorosulfonic acid (2 mL) in an ice bath; the reaction mixture was then warmed to room temperature gradually and stirred for 2 h after the addition. The reaction solution was cautiously added to ice-water (15 mL), and the aqueous mixture was extracted with dichloromethane (4 x 50 ml). The combined extracts were dried over anhydrous Na_2SO_4 and evaporated under a vacuum to give the required crude sulfonyl chloride (**3**). After evaporation of the solvent, the crude product was purified by column chromatography using methylene chloride / methanol (50:1) as the eluent to produce the product as a yellow solid.

1-[(para-chlorosulfonylphenyl)-3-methyl-5-methylsulfonyl]-1H-pyrazolo[4,3-e][1,2,4]triazine(**3**): Yield 65%. Melting point: 173-175 ^oC;^1H NMR (400 MHz, $CDCl_3$) δ:^1H NMR ($CDCl_3$) δ: 2.90 (s, 3H), 3.62 (s, 3H), 8.29 (d, 2H, J = 9.2 Hz), 8.81 (d, 2H, J = 9.2 Hz); ^{13}C NMR ($CDCl_3$) δ: 11.52, 67.40, 120.15, 129.03, 129.65, 138.01, 142.08, 142.71, 147.33, 148.38, 162.28. HRMS (ESI, m/z) Calcd for $C_{12}H_{10}ClN_5O_4S_2$[M+] 387.8256. Found [M^+] 387.8260. Anal. Calcd for $C_{12}H_{10}ClN_5O_4S_2$: C, 45.72; H, 3.84; N, 19.04. Found: C, 45.60; H, 3.90; N, 18.90.

Synthesis of sulfonamides (4a-b)

Derivative **3** (194 mg, 0.5 mmol) was dissolved in anhydrous acetonitrile (5 mL) and appropriate L -proline methyl ester or cis-4-hydroxy-L -proline methyl ester hydrochloride (1.75 mmol) was added. Then, sodium bicarbonate (147 mg, 1.75 mmol) was added to the resulting mixture. The reaction was stirred overnight at room temperature, and then the reaction mixture was concentrated *in vacuo* to afford the crude sulfonamide, as a yellow solid. The residue was purified on silica gel using a mixture of CH_2Cl_2:EtOH (25:1) as eluent to give the titled compounds as a yellow solid.

Methyl 1-[4-(3-methyl-5-methylsulfonyl-1H-pyrazolo[4,3-e][1,2,4]triazin-1-yl)phenylsulfonyl]pyrrolidine-2- carboxylate (4a): Yield 96%. Melting point: 79-83^{o}C;^1H NMR ($CDCl_3$) δ: 1.81-1.90 (m, 1H), 1.96-2.07 (m, 2H), 2.09-2.17 (m, 1H), 2.88 (s, 3H), 3.39-3.45 (m, 1H), 3.48-3.53 (m, 1H), 3.60 (s, 3H), 3.73 (s, 3H), 4.40 (dd, 1H,J_1 = 8.7 Hz, J_2 = 3,3 Hz), 8.09 (d, 2H, J = 8.7 Hz), 8.63 (d, 2H, J = 8.7 Hz);^{13}C NMR ($CDCl_3$) δ: 11.44, 24.68, 30.93, 40.76, 48.35, 52.46, 60.43, 119.96, 129.17, 137.12, 140.92, 146.50, 148.03, 161.93, 172.36. HRMS (ESI, m/z) Calcd for $C_{18}H_{20}N_6O_6S_2$[M^++H] 481.0885. Found [M^++H] 480.0880. Anal. Calcd for $C_{18}H_{20}N_6O_6S_2$: C, 44.99; H, 4.20; N, 17.49. Found: C, 44.81; H, 4.39; N, 17.32.

Methyl 4-hydroxy-1-[4-(3-methyl-5-methylsulfonyl-1H-pyrazolo[4,3-e][1,2,4]triazin-1-yl)phenylsulfonyl]pyrroli 2-carboxylate (4b): Yield 96%. Melting point: 112-114^o C;^1H NMR (acetone) δ: 2.07-2.13 (m, 1H), 2.15-2.21 (m, 1H), 2.85 (s, 3H), 3.45 (dt, 1H, J_1 = 11.2 Hz,J_2 = 1.7 Hz), 3.59 (s, 3H), 3.65 (dd, 1H,J_1 = 10.8 Hz, J_2 = 4.2 Hz), 3.73 (s, 3H), 4.36 (t, 1H, J = 7.9 Hz), 4.42 (bs, 1H), 8.16 (d, 2H, J = 8.7 Hz), 8.67 (d, 2H, J = 9.1 Hz);^{13}C NMR (acetone) δ: 11.26, 23.33, 40.21, 41.15, 57.60, 60.83, 70.11, 120.80, 130.32, 137.24, 142.19, 147.31, 149.60, 162.92, 173.10. HRMS (ESI, m/z) Calcd for $C_{18}H_{20}N_6O_7S_2$[M^++H] 497.0908 Found [M^++H] 497.0904. Anal. Calcd for $C_{18}H_{20}N_6O_7S_2$: C, 43.54; H, 4.06; N, 16.93. Found: C, 43.60; H, 4.15; N, 16.78.

Synthesis of tricyclic sulfonamides (**5a-b** -

MMs)

Sulfonamide derivative with a methylsulfonyl group (0.33 mmole) was dissolved in anhydrous ethanol (5 mL), and sodium azide (21 mg, 0.33 mmole) was added. The reaction mixture was refluxed until the substrate disappeared (control TLC). Then, the solvent was evaporated and the crude product was purified using column chromatography and CH_2Cl_2: MeOH (50:1) mixture as eluent to give the final compounds as a yellow solid.

Methyl 1-[4-(7-methyl-5H-pyrazolo[4,3-e]tetrazolo[4,5-b][1,2,4]triazin-5-yl)phenylsulfonyl]pyrrolidine-2-carboxylate (5a-MM-128): Yield 93%, Melting point: 125-127^{o}C;^1H NMR ($CDCl_3$) δ: 1.82-188 (m, 1H), 1.95-2.05 (m, 2H), 2.10-2.15 (m, 1H), 2.88 (s, 3H), 3.38-3.45 (m, 1H), 3.48-3.54 (m, 1H), 3.60 (s, 3H), 4.39-4.42 (dd, 1H, J_1 = 8.8 Hz, J_2 = 4.0 Hz), 8.09 (d, 2H, J = 8.8 Hz), 8.63 (d, 2H, J = 8.8 Hz); ^{13}C NMR ($CDCl_3$) δ: 11.43, 24.68, 30.92, 40.76, 48.34, 52.46, 119.95, 129.17, 137.11, 140.92, 146.50, 148.02, 161.92,

172.35. HRMS (ESI, m/z) Calcd for $C_{17}H_{17}N_9O_4S$ [M^++H] 444.11242. Found [M^++H] 444.11972. Anal. Calcd for $C_{17}H_{17}N_9O_4S$: C, 46.05; H, 3.86; N, 28.43. Found: C, 45.81; H, 4.03; N, 28.29.

Methyl 4-hydroxy-1-[4-(7-methyl-5H-pyrazolo[4,3-e]tetrazolo[4,5-b][1,2,4]-triazin-5-yl)phenylsulfonyl]-pyrrolidine-2-carboxylate (5b-MM-129): Yield 83%. Melting point: 122-128°C;^{1}H NMR (acetone) δ: 2.04-2.18 (m, 2H), 3.39 (d, 1H, J = 11.2 Hz), 2.85 (s, 3H), 3.62 (d, 1H, J = 12 Hz), 3.77 (s, 3H), 4.33 (d, 2H, J = 8.3 Hz), 4.57 (bs, 1H, OH), 8.08 (d, 2H, J = 9.2 Hz), 8.48 (d, 2H, J = 9.2 Hz); ^{13}C NMR (acetone) δ: 11.26, 41.14, 52.53, 57.59, 60.82, 70.22, 120.79, 130.32, 137.23, 142.19, 147.30, 149.60, 162.92, 173.09. HRMS (ESI, m/z) Calcd for $C_{17}H_{17}N_9O_5S$ [M^++H] 459.10734. Found [M^++H] 460.11469. Anal. Calcd for $C_{17}H_{17}N_9O_4S$: C, 44.44; H, 3.73; N, 27.44. Found: C, 44.35; H, 3.89; N, 27.20.

Cell culture

Human colorectal adenocarcinoma cell line DLD-1 (CCL-221) and HT-29 (HTB-38) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The DLD-1 line histologically is the most similar to a primary tumor. Line HT-29, in turn, is used to assess multidrug resistance, absorption of nutrients and chemically induced differentiation of enterocytes. Cells were cultured in RPMI 1640 medium (Sigma) and McCoy's 5a medium (ATCC), respectively, complemented with 10% of fetal bovine serum (FBS) and 1% of antibiotics: penicillin/streptomycin. The cells were maintained in an incubator, which provides optimal growth conditions for cell culture: 5% CO_2, 37°C, and humidity in a range of 90-95%. The cells were cultured in 100 mm plates (Sarstedt, USA). Subsequently, after obtaining a subconfluent cell culture, the cells were detached with 0.05% trypsin and 0.02% EDTA phosphate buffered saline without calcium and magnesium (Corning). Then, utilizing a hemocytometer, the number of cells was quantified and seeded at a density of 5 x 10^5 cells per well in six-well plates ("Nunc") in 2 mL of the growth medium (RPMI 1640 and McCoy's 5a). In the present study, cells that obtained 80% of confluency were used.

Cell viability assay

Cytotoxicity of newly synthesized derivatives was estimated by MTT assay as we described in an earlier article (Tankiewicz-Kwedlo, Hermanowicz, et al., 2017). Briefly, DLD-1 and HT-29 cells were seeded in six-well plates "Nunc" at a density of 5x10^5 cells/well and incubated for 24 hours in optimal growth conditions. Subsequently, the tested compounds, MMs, and reference drugs, roscovitine, 5-flurouracil, at concentrations 1, 2, 5, and 10 μM were added in duplicate and the plates were incubated for another 24 h. Next, the plates were washed with PBS three times. Then, 1 mL PBS and 50 μL of 5mg/cm^3 MTT solution were added and the incubation was continued for 4 hours. MTT tetrazolium is converted in viable cells into purple crystals of formazan. The conversion does not occur in dead cells. After the required time, the supernatant was removed and formazan crystals were dissolve in DMSO. The absorbance was measured at a wavelength 570 nm. The absorbance result obtained in the control was taken as 100% and the viability of the cells incubated with the tested compounds was showed as a percentage of the control cells.

[^{3}H]thymidine incorporation assay

DLD-1 and HT-29 cells were cultured in 6-well plates and treated with various concentrations (1 μM, 2 μM, 5^{o}μM, 10 μM) of the tested compounds and reference drugs, roscovitine and 5-flurouracil, for 24 hours. Following the incubation, the cells were treated with 0.5 μCi of radioactive [^{3}H]thymidine (specific activity 6.7 Ci/mmol) for 4 hours in optimal growth condition. Afterward, the medium was removed and cells were washed two times with 1 mL of 0.05 M Tris-HCl buffer containing 0.11 M NaCl. In the next step, the cells were washed twice with 1 mL of 5% TCA acid and then dissolved in 1 mL of 0.1 M NaOH containing 1% SDS. Following 5 minutes of incubation, the obtained cell lysates were transferred to scintillation vials previously filled with 2 mL scintillation fluid. The radioactivity was quantified on Scintillation Counter (Packard Tri-Carb Liquid Scintillation Counter 1900 TR, Perkin Elmer, Inc., Waltham, MA, USA). [^{3}H]thymidine uptake was expressed as dpm/well. The intensity of DNA biosynthesis in the control was taken as 100%. The values from the samples were presented as a percentage of the control cells.

Flow cytometry assessment of Annexin V binding

Induction of apoptosis was examined using Apoptosis Detection Kit II analyzed using a flow cytometry, as previously described (Tankiewicz-Kwedlo, Hermanowicz, et al., 2018). During programmed cell death, the phosphatidyloserine (PS) is transferred from the inner cell membrane to the cell surface. DLD-1 and HT-29 cells were incubated (24 hours) with the tested compounds MM-128, MM-129, and with the reference drugs: roscovitine and 5-flurouracil. All compounds were used in two concentrations: 1.5 μM and 3.0 μM. Following incubation, the cells were dyed with FITC-labeled annexin V and propidium iodide. It allows to identify viable, necrotic, early, and late apoptotic cells. The positive controls were cells in which apoptosis was induced by the addition of 2 μl of 3% formaldehyde. The cells were placed in a refrigerator for 15 minutes to induce apoptosis. Three controls were made: the first contained control cells and propidium iodide; the second, control cells and annexin V-FITC; and the third, control cells and propidium iodine and annexin V-FITC. Cells cultured in medium without the tested compounds were used as the negative control. The experiment was performed using the BD FACSCanto II flow cytometer and the results were parsed with FACSDiva software (BD Biosciences Systems, San Jose, CA).

Analysis of mitochondrial membrane potential

Changes in the mitochondrial membrane potential was provided with flow cytometry using JC-1 MitoScreen kit (BD Biosciences, USA). In normal cells, JC-1 (1,1',3,3'-tetraethyl-5,5',6,6'-tetrachloroimidacarbocyanine iodide) aggregates in the mitochondrial matrix. Nevertheless, in apoptotic and necrotic cells, this lipophilic dye diffuses out of mitochondria and stains cells with a green fluorescent. DLD-1 and HT-29 colon cancer cell lines covering about 80% of the plate were incubated with the novel synthesized compounds, MM-128, MM-129, and the reference drugs, roscovitine and 5-flurouracil, for 24 hours in an incubator at 37°C, 5% CO_2. All compounds were used at 1.5 μM and 3.0 μM concentrations. Following incubation, the medium was removed and the cells were washed two times with the required buffer. Then, the cells were suspended in a 10 μg/ml JC-1 dye and incubated in the dark for 15 minutes. The cells were washed with PBS and analyzed using BD FacsCanto II flow cytometer. The results were assessed using BD FacsDiva software (BD Biosciences Systems, San Jose, CA).

Caspase activity assays

Detection of caspase-8, -9, -10, -3/7 activity was assessed with the appropriate kit (caspase-8: FLICA Caspase-8 Assay Kit, caspase-9: FLICA Caspase-9 Assay Kit, caspase-10: FLICA Caspase-10 Assay Kit, caspase-3/7: FLICA Caspase-3/7 Assay Kit). DLD-1 and HT-29 were incubated for 24 hours with the tested MMs compounds and reference drugs, roscovitine and 5-flurouracil, in two concentrations: 1.5 μM and 3 μM. Following incubation, the cells were washed twice with cold PBS. Subsequently, the cells were resuspended in the required buffer (93 μl was gently mixed with 5 μl required FLICA and 2 μl Hoechst 33342) and incubated at 37 °C for 60 minutes. Then, the cells were washed twice with Apoptosis wash buffer and centrifuged at 300 x g. After preparing, the samples, the cells were resuspended in 100 μl buffer and labeled with 10 μg/ml propidium iodide. The experiment was performed using the BD FACSCanto II flow cytometer and the results were parsed with FACSDiva software (BD Biosciences Systems, San Jose, CA).

Zebrafish drug-screening assay

The zebrafish (*Danio rerio*) were maintained at 28.5°C in E3 buffer in 30 L aquaria at a rate of 1 fish per liter of water with cycles of 14/10 hours of light/darkness and fed in accordance with the guidelines established by the Research Animals Department of the RSPCA. The use of animals in scientific research in Europe is governed by the Directive 2010/63/EU of 22 September 2010. According to EU Directive earliest life-stages of zebrafish (embryo and eleutheroembryo cultures) are regarded as equivalent to *in vitro* cell culture therefore, do not fall into the regulatory frameworks dealing with animal experiments. In contrast, experiments with the free-feeding larvae older than 120h of development are classified as animal experiments and require permission. The rationale is that active hunting reflects a perception of environmental stimuli that go beyond simple reflexes. This is taken as an indication of a mature nervous system that controls behaviour. In our experiment we used zebrafish larvae younger than 120 hpf (hours post-fertilization)

therefore ethic approval was not required. Zebrafish embryos were obtained from mating adults, maintained and raised as described previously (Westerfield, 2000; Strahle, Scholz, et al., 2012). Zygote period cleaving eggs were transferred to 6-well plastic cell culture plates filled with embryo medium E3. The eggs (10–12 per well) were exposed to roscovitine (50 μM) (Li, Jiang, et. al., 2011), 5-FU (50 μM) (Xu, Hu, et al., 2019) and MM-129 (10 μM) for 3 h. The final volume of the medium in each well was 2 mL. DMSO was used as a drug solvent. The final concentration of DMSO in the wells did not exceed the damaging concentration of above 0.1%. The mock control embryos were incubated in embryo medium in the presence of 0.1% DMSO. The drug effect was recognized when all the eggs from one well changed in the same characteristic manner. Each experiment was carried out in three independent experiments. Observations of cell division and development of the zebrafish eggs were carried out using SteREO Discovery.V8 stereo microscope (Zeiss, Jena, Germany) once every 15 min within the first three hours of incubation.

Transfection

A day before transfection, $5x10^4$ cells were seeded per well in 24-well plate. HT-29 and DLD-1 cell lines were transfected with Lipofectamine 3000 (Invitrogen). Transfection was performed using 1000ng of the plasmid DNA (pmR-mCherry Vector, Clontech, Cat No 632542), 1.5 μL Lipofectamine®3000 Reagent and 1 μL P3000 Reagent per well, following the manufacture's protocol. The cells were selected 48h upon transfection for another 2 weeks in 1 mg/ml of Geneticin (G418) (ThermoFisher).

Zebrafish handling, establishment of xenograft

Colon cancer DLD-1 and HT-29 cells were transfected before transplantation. The zebrafish embryos were manually dechorionated 36 hours post-fertilization (hpf), and after another 12 hours were anesthetized by placing in 0.04 mg/mL ethyl 3-aminobenzoate met-hanesulfonate tricaine, which is a water soluble, fast-acting anesthetic agent. Zebrafish embryos were then transfer to a thin film of low-melting-point agarose to stabilize the fish in a lateral position. Colon cancer cells were loaded into a borosilicate glass needle pulled by a P-1000 Next Generation Micropipette Puller (Sutter Instrument Company). A suspension containing about 100–200 cells was injected into the inferior section of the yolk sac in a single injection by using an electronically regulated air-pressure microinjector (Narishige IM-300 Microinjector). After injection, the zebrafish were washed once with fish water and transferred to 6-well plate containing 2 mL of fresh fish water. DLD-1 and HT-29-xenografts (72 hpf) were incubated at 33°C with MM-129 (10 μM) and 5-fluorouracil (50 μM) and a combination of these drugs for 48 hours. Although embryos are normally allowed to develop at 28.5°C and human cells at 37°C, a compromise at 33°C works well.

Microscope imaging

Living zebrafish embryos were anesthetized with tricaine and embedded in a lateral orientation. The animals were analyzed for cytoplasmic fluorescence intensity. Images of the cells were acquired using an EVOS M5000 Imaging System with the following filters: GFP (470/22 nm Excitation; 510/42 nm Emission) and Texas Red (585/29 nm Excitation; 624/40 nm Emission). Image analysis was performed using ImageJ v1.51 software (National Institute of Health, USA).

Statistical analysis

Shapiro-Wilk's test of normality was used for data distribution analysis. The normally distributed data were expressed as mean ± SD. Multiple group comparisons were performed by one-way analysis of variance (ANOVA), and significant differences between the groups were assessed using the Tukey-Kramer test or the non-parametric Mann-Whitney U-test. Calculations were performed using GraphPad 6 Prism software. The differences were deemed statistically significant when $p<0.05$. Measurement of fluorescence intensity was analyzed using Image J 1.50a software.

RESULTS

Synthesis of novel 1,2,4-triazine derivatives

The synthesis of target tricyclic sulfonamides (MMs) was achieved by a convenient multiple procedure starting from the known 3-methyl-5-methylsulfanyl-1-phenyl-1*H* -pyrazolo[4,3-*e*][1,2,4]triazine (**1**), which was subjected to an oxidation process under phase transfer catalysis to receive the corresponding sulfone **2** in excellent yield (Scheme 1) (Mojzych, & Rykowski, 2004). The chlorosulfonylation reaction of compound **2** in neat chlorosulfonic acid at 0°C proceeded smoothly and selectively at the 4'-position of the phenyl ring to give the desired product **3** in good yield. Next, the chlorosulfonyl derivative**3** was readily coupled L-proline derivatives in acetonitrile at room temperature to produce sulfonamides **4a-4b** that were reacted with sodium azide in absolute ethanol under reflux to furnish final tricyclic products MMs (**5a-5b**). The structures and the purity of the newly synthesized compounds were characterized using the ^{1}H and ^{13}C NMR, and HRMS methods together with elemental analysis.

Biological activity of novel 1,2,4-triazine derivatives

The effect of pyrazolo[4,3-*e*]tetrazolo[4,5-*b*][1,2,4]triazine derivatives on the viability of colon adenocarcinoma cell lines DLD-1 and HT-29 was assessed with MTT assay. The study showed that the tested compounds have a much higher cytotoxic potency than the reference drugs in both cell lines. In DLD-1 cells, the IC_{50} values were: 1.1 μM for MM-128, and 3.1 μM for MM-129, while for roscovitine and 5-fluorouracil, it was above 10 μM. In HT-29 cells, the IC_{50} values were: 1.7 μM for MM-128, 3.1 μM for MM-129 and above 10 μM for reference compounds (Fig 2A, 2B).

To confirm the antiproliferative effects of the new synthesized compounds, their effect on DNA biosynthesis in DLD-1 and HT-29 colon adenocarcinoma cells was investigated. The estimation was made by measuring the incorporation of radioactive labeled thymidine into the DNA of the cancer cells after 24-hour incubation with various concentrations of the tested and reference compounds (1 μM, 2 μM, 5 μM, 10 μM). The results are presented in Figures 2C, 2D. MM-128, and MM-129 were able to inhibit the growth of human colon adenocarcinoma cell lines in a dose dependent manner. MM-128 showed the highest inhibitory effect against DLD-1 cells, while MM-129 against HT-29 cell line. The concentration of MM-128 needed to inhibit the incorporation of [^{3}H]thymidine into DNA in DLD-1 cells by 50% (IC_{50}) was 0.6 μM, whilst for MM-129 in HT-29 was 2.3 μM. This effect was stronger than that of roscovitine and 5-fluorouracil in both cell lines.

MMs induce apoptosis through phosphatidylserine externalization and loss of mitochondrial membrane potential

Determination of the apoptosis status of DLD-1 and HT-29 cells after 24 hours of incubation with 1,2,4-triazine derivatives, roscovitine, and 5-flurouracil was assessed by dual annexin V and iodium propidium staining using flow cytometry. One of the characteristic early changes in the cells during programmed cell death is displacement of phosphatidylserine from the internal to the external cell membrane – externalization of phosphatidylserine. Dual staining allows to detect live cells (not binding annexin V and PI), early-apoptotic cells (binding annexin V and not binding PI), late-apoptotic cells (binding annexin V and PI), and necrotic cells (binding PI and not binding annexin V). Following 24 h incubation with the studied compounds, the activation of programmed cell death in DLD-1 and HT-29 cell lines was observed (Fig 3A, 3B). A total of 6.1% of apoptotic and 1.9% necrotic cells were observed in the population of DLD-1 control cells. The strongest proapoptotic properties on this cell line were exhibited by the MM-129 compound at a concentration of 3 μM, where the percentage of early and late apoptotic cells was about 82.5%. In the HT-29, the highest number of apoptotic cells was also in response to MM-129 (3 μM) 38.9% vs 12,7% and 12,6% in cells treated with roscovitine or 5-fluorouracil and 7.7% in control cells These data reveal that the cytotoxic activity of pyrazolo[4,3-*e*]tetrazolo[4,5-*b*][1,2,4]triazine derivatives against DLD-1 and HT-29 cancer cells is due to the induction of programmed cell death.

A decrease in mitochondrial membrane potential (MMP) is one of the earliest changes associated with programmed cell death. During apoptosis, the permeability of the external and the internal mitochondrial membrane is increased. As a consequence, mitochondrial proteins are released into the cytosol through the outer membrane e.g. cytochrome c and apoptosis inducing factor (AIF). The exposure to MM-128, MM-129,

roscovitine and 5-fluorouracil for 24 h resulted in the loss of membrane integrity in both colon adenocarcinoma cell lines. The rate of MMP-disrupted cells was increased in a dose dependent manner – Figures 4A, 4B. Percentage of cells with decreased levels of MMP after MM-129 (3 μM) exposure was 71.3% and 81% for DLD-1 and HT-29, respectively. This suggests that pyrazolo[4,3-*e*]tetrazolo[4,5-*b*][1,2,4]triazine-induced MMP disruption involved in the intrinsic apoptosis pathway.

MMs increase caspases activity

Caspases play a major role in the activation of apoptosis. There are two main pathways that can lead to the activation of caspases: the internal (mitochondrial) and the external (receptor) pathway. The first path leads to mitochondrial membrane perturbation and release of cytochrome C to the cytosol. This protein binds to the Apaf-1 and procaspase-9 in the cytoplasm creating a multiprotein complex-apoptosome, which activates executive caspases. Hence, the effect on the activation of caspase-9 by novel pyrazolo[4,3-*e*]tetrazolo[4,5-*b*][1,2,4]triazine derivatives was examined after 24 h incubation with MM-128, MM-129, roscovitine or 5-flurouracil (supplemental Figure 1S). The results revealed that the new compounds led to an increased expression of caspase-9 in both cell lines. In the DLD-1 cell culture, compound MM-128 at a 3 μM concentration showed a stronger effect on the activation of caspase-9 than the reference drugs. In this case, the percentage of cells with the active form of this initiator caspase was 84.6%. In the HT-29 cell line, 36.4% of cells had an activated initiation of caspase-9 after 24 h incubation with MM-129 at the same concentration.

Stimulation of the extrinsic pathway starts with the activation of death receptors. After binding the appropriate death ligand, the receptors accumulate in clusters in the cell membrane and promote the recruitment of adapter proteins. These proteins have the death effector domain (DED), through which they interact with procaspase-8 and procaspase-10. The receptor, adapter protein, and procaspase form the complex DISC, that led to the activation of caspase-8 and caspase-10, and subsequently cell death. The activation of caspase-8 and caspase-10 was determined after 24-hour treatment with MMs, ROS and 5-FU by FLICA Caspase-8 Assay Kit (supplemental Fig 2S) and FLICA Caspase-10 Assay Kit (supplemental Fig 3S). It was shown that all the tested compounds increased the expression of the active form of caspase-8 and caspase-10 in both cell lines. The highest percent of cells with the active form of caspase 8 was observed for MM-128 at a 3 μM concentration, and it was 89.5% on DLD-1 cell line. In the case of HT-29, two compounds represented a similar effectiveness: 68.0% and 67.8% of cells were detected with the active form of caspase-8 after 24-hour treatment with MM-128 and MM-129, respectively. Similar effect on the activation of caspase-10 in DLD-1 was also exhibited by compounds MM-128 and MM-129 at a 3 μM concentration; the percentage of cells with the active form of initiator caspase-10 was 43.0% and 49% respectively. The highest percentage of HT-29 cells expressing active caspase-10 was evoked by compound MM-129, and it was 37.9% (supplemental Fig 3S).

The caspases-3/7 belongs to the group of executive caspases. Procaspases-3/7 are cleaved into their active forms, caspase-3 and caspase-7, by activated caspases-8, -10, and -9. Using flow cytometry, it was examined whether the process of programmed cell death in the studied cell lines initiated by new pyrazolo[4,3-*e*]tetrazolo[4,5-*b*][1,2,4]triazine derivatives and reference compounds is related with caspase-3/7. The obtained results showed that all the tested compounds increased the expression of the active form of caspases-3/7 in DLD-1 and HT-29 cell lines (Fig 5). The strongest effect was observed in cells incubated in the presence of MM-128 at a 3 μM concentration in DLD-1 and MM-129 at a 3 μM concentration in HT-29. The percentage of cells with active executive caspase-3/7 was 81.0% on DLD-1 and 57.9% on HT-29 cells, respectively.

Anti-proliferative activity of MM-129 in zebrafish model

The antiproliferative activity of MM-129 and the reference drugs (roscovitine, 5-flurouracil) were also examined in a zebrafish embryo model (Fig 6). At a constant temperature of 28.5°C (ST), the ZF egg cleaves first at 45 min post fertilization forming the two-cell stage egg. Zebrafish embryos reach 4-cell, 8-cell, and

64-cell stages within 1, 1.25, and 2 hours post fertilization (hpf), respectively. Subsequently, they entering into the blastula stage (2.25–5.25 hpf), the gastrula stage (5.25–10 hpf), and the segmentation stage (10–24 hpf) to finally hatch out between 48 and 72 hpf (Uckun, Dibirdik, et al., 2007; Taung, Wu, et al., 2018). In untreated eggs, we observed normal embryo development revealed in consecutive synchronous cleavages. This process was disturbed in embryos exposed to MM-129, roscovitine and 5-FU. At 1 hpt (hour post-treatment), MM-129-treated eggs showed a deterioration of cell division, cell disorientation, and initial signs of cell fusion. Within the next 30 min, fusion dramatically progressed in MM-129-treated eggs, while the control eggs continued cell division without any apparent delay. When zebrafish eggs were treated with roscovitine and 5-FU, they developed normally for 1h up to the 4-cell stage, and then at the 8-cell stage they showed proliferation arrest. After two hours of incubation, we noticed complete cell fusion and lysis in groups exposed to MM-129 and roscovitine. 5-FU did not show such strong changes during the study period. No alteration in cell division and development in untreated control eggs was observed.

MM-129 inhibits tumor development in zebrafish xenografts

DLD-1 and HT-29 cells stably transfected with a plasmid encoding mCherry fluorescent protein (pmR-mCherry) were implanted into the yolk sac of wild-type (WT) zebrafish 48 hpf embryos. The scheme of the experimental protocol is presented in Figure 7A. Three days after cell injection, solid tumors were established in 100% of DLD-1 (n = 16) and HT-29 xenografts (n = 16). The embryos were then incubated with MM-129, 5-fluorouracil, or a combination of these compounds for 48 hours. The control group received the MM-129 solvent (0.1% DMSO/PBS). In both DLD-1 and HT-29 xenografts, we observed a significant reduction in tumor development in the MM-129 treated group compared with the control group, as reflected by the drop in fluorescence intensity (62.8+-2.76 vs. 90.19+-10.86 and 57.39+-17.1 vs. 101.4+-6.67, respectively; *p<0.05) (Fig 7B, 7C). Incubation with MM-129+5-FU significantly reduced the number of cancer cells in both DLD-1 (45.09+-12.66) and HT-29 (25.92+-8.43) xenografts compared with the appropriate control (90.19+-10.86 and 101.4+-6.67; ***p<0.001). The combination of MM-129+5-FU showed more pronounced anticancer activity than 5-FU alone in both DLD-1 (82.65+-17.04, #p<0.05) and HT-29 cells (70.21+-20.78, #p<0.05). Similarly, the combination of these compounds was also more effective in reducing cancer cells than MM-129 alone in DLD-1 (62.8+-2.76, NS), but this effect was statistically significant only in HT-29 xenografts (57.39+-17.1, ^p<0.05). These results indicate that MM-129 showed a markedly synergistic anticancer effect when used in combination with 5-FU.

DISCUSSION

Roscovitine, (2*R*)-2-{[6-(benzylamino)-9-isopropyl-9*H* -purin-2-yl]amino}-1-butanol also called CY-202 or seliciclib, is a low molecular weight purine derivative with a characteristic ring structure. It belongs to cyclin-dependent kinase (CDK) protein inhibitors, CDKs play a key role in regulating the cell cycle, promoting its progression or transition between the individual phases (Dorand, Nthale, et. al., 2016; Cortez, Ivan, et al., 2015). Roscovitine has been shown to possess anticancer activity in various *in vitro* and *in vivo* models (Aldoss, Tashi, et al., 2009; Guzi, 2004). The antitumor action of roscovitine has been tested using cancer xenografts of LoVo human colorectal cancer cells, MESSA-DX5 human uterine carcinoma cells, or MDA-MB 231 human breast cancer cells grafted into CD1 nude mice (Mcclue, Blake, et al., 2002; Maggiorella, Deutsch, et al., 2003).

The aim of the present study was to evaluate the anticancer potential of new pyrazolo[4,3-*e*]tetrazolo[4,5-*b*][1,2,4]triazine sulfonamides (MM-128, MM-129), which may be found applicable in the treatment of patients with colorectal carcinoma. Undoubtedly, this kind of cancer is a significant problem, both from the medical and social point of view. For this purpose were conducted preclinical studies of new 1,2,4-triazine derivatives both *in vitro* and in a zebrafish cancer model.

We first tested the synthesized compounds for their effects on cell survival using DLD-1 and HT-29 cell lines. Cytotoxicity assay revealed that the novel MMs compounds were more effective than hitherto used agents, including roscovitine and reference drug 5-fluorouracil. They statistically significantly reduced cell viability and inhibited DNA biosynthesis of DLD-1 and HT-29. The fact that they have high cytotoxic activity at a low

micromolar level is important. The available evidence indicates dose-dependent cytotoxicity of roscovitine with IC_{50} ranging from 15 to 25μM in multiple myeloma cells, from 18 to 22 μM in cervical carcinoma (C33A, HCE-1, HeLa, SiHa) and 67.55 μM in human glioblastoma A172 (Raje N, Kumar, et al., 2005; Cui, Wang et al., 2013; Kolodziej, Goetz, et al., 2015). Similar data exist for 5-fluorouracil, which induces significant colorectal cancer cell growth inhibition at a much higher concentration of 50 μM (Mhaidat, Bouklihacene, et al., 2014).

In our study, the IC_{50} values for roscovitine and 5-FU were at least 3-fold higher than MMs (1,1-3,1 μM). New 1,2,4-triazine derivatives displayed a significantly higher potency compared with the currently used compounds as well as other triazine derivatives. 1,2,4-triazine derivatives bearing thiophene moieties were tested for their cytotoxic effect against Hep-G2, MCF-7, and HCT-116. Their impact on the viability of cells after 48 h incubation were assessed using paclitaxol as a reference drug and they presented IC_{50}values in the range of 8.79-36.41 μM (Saad, Youssef, et al., 2011). Other 1,2,4 triazine derivatives were screened by the NCI for their*in vitro* antitumor activity. The strongest compound showed antiproliferative activity against the 60 NCI human tumor cell lines, with GI_{50} ranging from 1.47 to 12.2 μM, more potent than 5-fluorouracil used as a reference drug.

In this study, the antiproliferative and antitumor activity one of the new 1,2,4-triazine derivatives was confirmed in a zebrafish (ZF) embryo model. High genetic and physiological similarities with humans cause that zebrafish are often used as a strong and cost-effective animal model for cancer research and the discovery of cancer drugs. The small size, transparency of zebrafish embryos, easy manipulation, short testing period, and small amount of tested drugs are undoubtedly important aspects giving zebrafish an advantage over the mouse/rat model of cancer. A preliminary *in vivo* screening test showed that MM-129 possesses the most biological activity, therefore we decided to use it for further studies. This compound blocked cell division in a zebrafish embryo model at the 4-cell stage of embryonic, development and these disorders were noticeable earlier than in the case of roscovitine or 5-FU. Two hours of incubation led to complete cell fusion and lysis in groups exposed to MM-129. Cell division blockade and developmental arrest caused by this compound were irreversible.

The anticancer action of MM-129 was also investigated in a zebrafish embryo xenograft model. The xenograft experiment using DLD-1 and HT-29 human colorectal cancer cells grafted into the zebrafish yolk showed a significant antitumor effect of MM-129 with a 30% (DLD-1 xenografts) and 43% (HT-29 xenografts) reduction in the number of cancer cells compared with the control group. A basic element of colon cancer treatment is surgery, which is aimed at obtaining intestinal tissue free of cancer. Chemotherapy complements surgical intervention and is based mainly on 5-FU. For chemo, either the FOLFOX (5-FU, leucovorin, and oxaliplatin) or CapeOx (capecitabine and oxaliplatin) regimens are used most often, but some patients may get 5-FU with leucovorin or capecitabine alone based on their age and health needs (NCCN Guidelines 2018). It should be emphasized that MM-129 turned out to be more potent in inhibiting tumor development compared with 5-FU in both DLD-1 and HT-29 xenografts. Furthermore, MM-129 clearly showed synergistic anticancer effects when used in combination with the latter.

Programmed cell death (PCD) is a crucial process in animal development and tissue homeostasis, responsible for the elimination of senescent, damaged, and unhealthy cells from the body (Fuchs & Steller, 2011). The mechanism of apoptosis is highly complex, multistage, and involves many pathways. Based on the mechanism of roscovitine action, which is widely described as an effective inducer of apoptosis and apoptosis-dependent cell death (Hep-G2, MCF-7, HCT-116), we assessed the impact of new 1,2,4-triazine derivatives on several apoptotic pathways (Kolodziej, Goetz, et al, 2015; Saad, Youssef, et al., 2011; Tirado, Mateo-Lozano, et al., 2005). As has been summarized in Figure 8, the tested compounds affected several cellular targets involved in the induction of apoptosis-dependent cell death. First, we noticed significant accumulation of apoptotic colon cancer cells with externalized PS after incubation with MMs. To explore the cellular mechanism underlying the synthesized compounds' induction of apoptosis, we examined the alterations of the mitochondrial transmembrane potential and caspase activity by using flow cytometry analysis. We found that all new derivatives increased the number of cells with decreased levels of MMP and contributed to a

marked increase in the activity of all tested caspases compared with the control cells in both lines.

Our results are in agreement with Jiang et al., who synthesized 3-amino-1,2,4-benzotriazine-1,4-dioxide derivatives and demonstrated that they determined apoptosis in cancer cells by inducing a loss of mitochondrial membrane potential (Jiang, Yang, et al., 2006). Mechanism studies of other 1,2,4-triazine derivatives bearing an extra 1,2,4-benzotriazine-1-oxide chromophore also revealed that the cytotoxic activity seems to be due to the activation of caspase 3/7 and G2/M arrest-mediated apoptosis (Lee, Huang., et al., 2014). Herein, roscovitine or 5-FU used at low doses had no effect on apoptosis. This is in line with previous reports indicating that 5-FU induces caspase-dependent apoptosis of CRC cells at doses of 50 μM (Mhaidat, Bouklihacene, et a., 2014). Roscovitine also exhibited a clear proapoptotic effect in the A172 and G28 only at 50 μM and 100 μM, respectively (Kolodziej, Goetz, et al, 2015). We intentionally used the same doses for all compounds, both tested and reference. Our results clearly indicate that new the 1,2,4-triazine derivatives are apoptosis-inducing factors with higher efficiency than roscovitine or 5-FU.

CONCLUSIONS

New 1,2,4-triazine derivatives are shown to exert potent antitumor activity on colorectal cancer. They possess high cytotoxic, antiproliferative and proapoptotic activity. Their effectiveness is higher compared with the standard chemotherapy for colorectal cancer, i.e. 5-fluorouracil. However, their cellular targets remain unknown and require further mechanistic investigation. The discovery of an innovative therapeutic option that improves the prospects of treating patients is currently a crucial challenge. There is a real chance that the results presented in this study will help in the development of a new, effective therapy, which could be an attractive alternative to the already existing methods of colon cancer treatment.

Authors' contributions

Conceptualization, J.M.H., A.B., D.P.; methodology, A.S., J.M.H., R.C., J.K., A.B., A.P.; software, B.S., A.S.; validation, J.M.H, A.S., M.M., R.C.; formal analysis, B.S., A.S.; investigation, A.S., R.C., J.M.H., B.S.; resources, J.M.H.; data curation, J.M.H., A.S., M.M.; writing—original draft preparation, J.M.H.; writing—review and editing, D.P., K.P., A.B., K.B., M.M.; visualization, A.B., K.B., B.S., A.S., R.C.; supervision, A.B., K.B., M.M., A.R.M., D.P., K.P.; project administration, J.M.H. D.P.; funding acquisition, D.P. All authors have read and agreed to the published version of the manuscript.

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Figure legend

Figure 1. Chemical structure of MM-128 and MM-129 (MMs).

Figure 2. Viability of DLD-1 (**A**) and HT-29 (**B**) colon cancer cells treated for 24 hours with different concentrations of MM-128, and MM-129, roscovitine (ROS) and 5-fluorouracil (5-FU), and the antiproliferative effects of the studied compounds in DLD-1 (**C**) and HT-29 (**D**) colon cancer cells measured by the inhibition of [^{3}H]thymidine incorporation into DNA. Mean values ±SD from three independent experiments (n = 3) done in duplicate are presented.

Figure 3. Representative flow cytometry dot-plots for Annexin V-FITC-Propidium iodine assay of DLD-1 (**A**) and HT-29 (**B**) colon cancer cells after 24 hours of incubation with MM-128 and MM-29, roscovitine (ROS) or 5-fluorouracil (5-FU) (1.5 μM and 3 μM).

Figure 4. Representative dot-plots presenting the loss of mitochondrial membrane potential (MMP) of DLD-1 (**A**) and HT-29 (**B**) colon cancer cells treated for 24 hours with MM-128, and MM-129 , roscovitine (ROS) or 5-fluorouracil (5-FU) (1.5 μM and 3 μM).

Figure 5. Flow cytometric analysis of populations DLD-1 (A) and HT-29 (B) colon cancer cells treated for 24 hours with MM-128, MM-129, roscovitine (ROS) or 5-fluorouracil (5-FU) (1.5 μM and 3 μM) for active caspase-3/7. Mean percentage values from three independent experiments (n = 3) done in duplicate are presented. ****p< 0.0001 vs. CON.

Figure 6. Effect of MM-129, 5-fluorouracil (5-FU), and roscovitine (ROS) on cell division in the zebrafish embryo. Zebrafish eggs after 0; 1; 1.25 and 2h of exposure to MM-129, 5-FU and ROS; n=10. hpf – hours post fertilization; hpt – hours post treatment.

Figure 7. Schematic of xenograft assay and analysis of tumor development (**A**). Site-specific injection (yolk sac) of transfected (red) colon cancer cells (DLD-1 and HT-29) into 48 hpf zebrafish embryos and imaging analysis of tumor growth after 48 hours of incubation with M-129 (50 μM), 5-FU (50 μM), or a combination of these agents (**B**). Quantification of total fluorescence by colon cancer cells 3 days after injection (**C**) n=4, *p<0.05, ***<0.001 vs. CON; ^ p<0.05 vs. MM-129 # p<0.05 vs. 5-FU.

Figure 8. Schematic representation of possible anticancer mechanisms of MMs. 1 - phosphatidylserine (PS) externalization; 2 - loss of mitochondrial membrane potential; 3 – activation of extrinsic pathway of apoptosis; 4- the activation of internal (mitochondrial) apoptosis; 5 – activation of executive caspases.

Apaf-1 - apoptotic protease activating factor 1; Bak- Bcl-2 - homologous antagonist/killer; Bax - Bcl-2-associated X protein, Bcl-2 – antiapoptotic protein; Bid - Bax-like BH3 protein; tBid - truncated BID; FADD - Fas-associated death domain protein; MMs - MM-128, MM-129

Scheme legend

Scheme 1. Reagents and conditions: (a) $KMnO_4$, CH_3COOH, Bu_4NBr, benzene-H_2O, rt, 1h; (b) $ClSO_3H$, 0°C to rt, 2h; (c) L-proline derivative hydrochlorides, MeCN, rt, 3h; (d) NaN_3, EtOH, reflux, 18h.

Supplementary material

Figure 1S. Flow cytometric analysis of populations DLD-1 (**A**) and HT-29 (**B**) colon cancer cells treated for 24 hours with MM-128, MM-129, roscovitine (ROS) or 5-fluorouracil (5-FU) (1.5 μM and 3 μM) for active caspase-9. Mean percentage values from three independent experiments (n = 3) done in duplicate are presented. ****p< 0.0001 vs. CON.

Figure 2S. Flow cytometric analysis of populations DLD-1 (**A**) and HT-29 (**B**) colon cancer cells treated for 24 hours with MM-128, MM-129, roscovitine (ROS) or 5-fluorouracil (5-FU) (1.5 μM and 3 μM) for active caspase-8. Mean percentage values from three independent experiments (n = 3) done in duplicate are presented. **p< 0.01; ****p< 0.0001 vs. CON.

Figure 3S. Flow cytometric analysis of populations DLD-1 (**A**) and HT-29 (**B**) colon cancer cells treated for 24 hours with MM-128, MM-129, roscovitine (ROS) or 5-fluorouraci (5-FU) (1.5 μM and 3 μM) for active

caspase-10. Mean percentage values from three independent experiments (n = 3) done in duplicate are presented. ***p< 0.001; ****p< 0.0001 vs. CON.

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