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In vitro investigation of the antiproliferative and proapoptotic effects of hyperatomarin – a bicyclic prenylated acylphloroglucinol from *Hypericum annulatum* Moris subsp. *annulatum* against human tumor and endothelial cells

Sefedin Biljali[1], Georgi Momekov[2,3]*, Paraskev Nedialkov[4], Dimitrina Zheleva-Dimitrova[4], Gerassim Kitanov[5], Denitsa Momekova[6], Nikolay Stoyanov[3,7], Margarita Guenova[3,7], Antoaneta Michova[3,7,8], Margarita Karaivanova[1]
Correspondence: gmomekov@cetrh.org
[2] Department of Pharmacology, Pharmacotherapy and Toxicology, Faculty of Pharmacy, Medical University - Sofia, Bulgaria
[3] Center of Excellence – Translational Research in Hematology, National Specialized Hospital for Active Treatment of Hematological Diseases, Sofia, Bulgaria.

Abstract

Background: Hyperatomarin is an acylphloroglucinol isolated from *H. annulatum Moris* subsp. *annulatum*, a species endemic for Sardinia and the Balkan Peninsula. Preliminary studies have shown antibacterial activity, serotonin re-uptake inhibition and cytotoxicity against tumor cell lines. In continuation of these studies we hereby report on the antiproliferative and proapoptotic potential of hyperatomarin against chemosensitive and resistant tumor cell lines and human umbilical vein endothelial cells (HUVECs).

Methods: The cytotoxic effects of hyperatomarin were tested in a panel of human tumor cell lines, including a multi-drug resistant model HL-60/Dox, using the MTT-dye reduction assay. The pro-apoptotic activity of hyperatomarin in HL-60 and KG-1 leukemic cells was investigated with a commercially available 'Cell Death Detection ELISA' kit. The effects of hyperatomarin on the cell cycle progression of KG-1 cells were studied by flow cytometric analysis following propidium iodide staining of cellular DNA. The angiostatic effects of the tested compound were evaluated in an in vitro angiogenesis assay using proliferating HUVECs.

Results: Hyperatomarin proved to be a potent cytotoxic and proapoptotic agent, against chemosensitive and multidrug-resistant tumor cell lines, and human endothelial cells stimulated to proliferate by VEGF treatment. Hyperatomarin treatment was found to induce G1 arrest in KG-1 cells and to induce apoptotic DNA-fragmentation, presumably via activation of the caspase signaling cascade.

Conclusions: Our data indicate that hyperatomarin has multimodal activity targeting tumor cells and abnormally proliferating endothelial cells. On these grounds hyperatomarin could be considered a promising drug candidate with hybrid cytotoxic and angiostatic properties necessitating further detailed pharmacological evaluation as antineoplastic agent.

Key words: hyperatomarin, *Hypericum annulatum Moris* subsp. *annulatum*, acylphloroglucinols, cytotoxicity, apoptosis, antiangiogenic effects

Background

Acylphloroglucinols comprise an important class of biologically active secondary metabolites peculiar for the plants from the related families Hypericaceae and Clusiaceae (Guttiferae) [1, 2]. The complex substitution patterns involving different acyl and isoprenoid functionalizations, glycosylation, oxidation, or cyclization of the phloroglucinol core structure conditions the tremendous structural diversity of these compounds [1-6]. Not surprisingly this chemical variety is translated into complex, multimodal pharmacological activities such as antibacterial [7-15], antiprotozoal [16-18], antifungal [17,19,20], antiinflammatory [10,21-26], psychopharmacological [4,27-31], among others. A feature of profound interest however that has recently driven much attention to the prenylated acylphloroglucinols is the established cytotoxic activity of representative compounds against tumor cell lines [13,15,18,19,24,25,32-42]. Moreover, some members of the acylphloroglucinol family have been found to suppress tumor-induced angiogenesis, using different *in vitro* models and read-out systems [38,43,44]. An exemplary antineoplastic acylphloroglucinol is hyperforin, a polyprenylated

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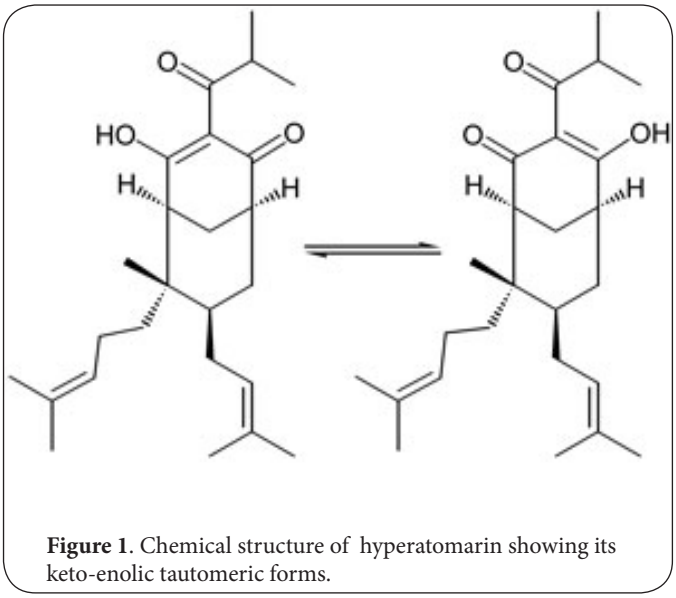

Figure 1. Chemical structure of hyperatomarin showing its keto-enolic tautomeric forms.

agent isolated from *Hypericum perforatum* (St. John's wort), which in addition to its antidepressant effects [3,29], immune modulatory and antiinflammatory effects [10,21,23-26,45,46], has been well documented to inhibit the growth of human tumor cells [11,33,47], to induce programmed cell death through apoptosis [36,41], and moreover to be a potent inhibitor of key events implicated in angiogenesis such as endothelial proliferation and migration [43], matrix metalloprotease activity, and *in vitro* microtubule formation [10,23,38,45,48,49]. On these grounds hyperforin is considered a promising anticancer drug candidate with combined cytotoxic and angiostatic properties [10,11].

Hyperatomarin (Figure 1) is a structurally related bicyclic prenylated acylphloroglucinol isolated from the aerial parts of *Hypericum annulatum* Moris subsp. *annulatum* [12], an endemic species inhabiting Sardinia and the Balkan Peninsula [3,5,50]. The compound has shown antibacterial activity [12] and has been demonstrated to modulate the reuptake of serotonin [51]. In a pilot oncopharmacological study hyperatomarin exerted prominent cytotoxic activity in a panel of human tumor cell lines and was found to induce apoptosis, applied at low micromolar concentrations [39].

In continuation of these studies we hereby report on the cytotoxicity of this compound in additional tumor models, including a multidrug-resistant cell line, its ability to induce programmed cell death and its effects on the cell cycle progression. Moreover the ability of hyperatomarin to suppress the proliferation of VEGF-stimulated human umbilical vein endothelial cells is described as well.

Methods

Materials, Reagents and Solutions

Formic acid, 2-propanol, and L-glutamine were purchased from AppliChem GmbH, (Darmstadt, Germany). Fetal calf serum (FCS), propidium iodide, rhuVEGF, and RPMI 1640 medium were purchased from Sigma – Aldrich GmbH (Steinheim, Germany). The cell culture flasks and the flat-bottomed multi-well plates were obtained from Nunc A/S (Kamstrupvej, Denmark). The tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was supplied from Merck (Darmstadt, Germany). The 'Cell death detection ELISA™' kit was purchased from Roche Diagnostics (Indianapolis, USA). The referent antineoplastic drug daunorubicin was used as a commercially available sterile dosage form for clinical application (Daunoblastin®, Farmacia, Italy). The endothelial cells EBM-2 growth medium and its supplementary Bullet Kit® growth factor mixture were purchased from Clonetics (Walkersville, MD, USA). Stock solutions of the tested compounds were freshly prepared in DMSO and promptly subset to serial dilution in RPMI-1640 to yield the desired working solutions. At the final concentrations obtained cells were never exposed to solvent concentrations exceeding 0.5%.

Plant material, extraction, isolation and identification of hyperatomarin

The aerial parts of *Hypericum annulatum* Moris *subsp. annulatum* were collected during the flowering season from a wild habitat, located at the central part of the Rhodope Mountains. A voucher specimen (No. 144296) has been deposited at the Herbarium of the Institute of Botany at the Bulgarian Academy of Sciences (SOM).

The detailed description of the extraction, isolation and identification of hyperatomarin has been previously reported [39]. In brief, air-dried and powdered plant material of *H.annulatum* Moris *subsp. annulatum* were refluxed exhaustively with *n*-hexane and then with methanol. Both extracts were separately evaporated under *vacuo*. The hexane extract was subjected to column chromatography on silica gel, eluted with mixtures of *n*-hexane-ethylacetate. The pooled fraction containing hyperatomarin was further purified by means of RP-18 column chromatography, using acetonitrile-water mixtures as eluent. Hyperatomarin was obtained as a colorless oil. The structure was confirmed by means of spectral methods (UV, IR, ^{1}H- and ^{13}C-NMR, EI-MS). The purity of the compound (^{3} 96%) was determined by HPLC.

Cell lines and culture conditions

The human tumor cell lines, used in this study namely KG-1, HL-60 (acute myeloid leukemias), HL-60/Dox (a multi-drug resistant variant, characterized by over expression of MRP-1), 5637 (urinary bladder cancer), MDA-MB-231 (ER-negative breast carcinoma) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ GmbH, Braunschweig, Germany). The cells were grown in controlled environment – cell culture flasks at 37°C in an incubator 'BB 16-Function Line' Heraeus (Kendro, Hanau, Germany) with humidified atmosphere and 5% CO_2. Cells were kept in *log* phase by

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supplementation with fresh medium, two or three times a week. The cancer cell lines were maintained in 90% RPMI-1640, supplemented with 10% FBS and 2mM L-glutamine. Leukemic cell lines were grown as suspension type cultures, whereas 5637 and MDA-MB-231 were maintained as monolayer cultures. Human umbilical vein endothelial cells (HUVECs) were supplied from Clonetics (Walkersville, MD, USA) and were grown in EBM-2 medium supplemented with a Bullet Kit® growth factor mixture, containing fetal calf serum, amphothericin B, and hydrocortisone, as supplied by the manufacturer (Clonetics, Walkersville, MD, USA). The details on their cell culture conditions are summarized in the angiogenesis protocol, below.

Cytotoxicity assessment (MTT-dye reduction assay)

The cellular viability was assessed using the MTT-dye reduction assay as described by Mosmann [52] with slight modifications [53]. The assay is based on the reduction of the yellow tetrazolium dye MTT to a violet formazan product *via* the mitochondrial succinate dehydrogenase in viable cells. In brief, exponentially growing cells were seeded in 96-well flat-bottomed microplates (100 µl/well) at a density of 1×10^5 cells per ml and after 24h incubation at 37°C they were exposed to various concentrations of the tested compounds for 48 h. For each concentration a set of at least 8 wells were used. After the exposure period 10µl MTT solution (10mg/ml in PBS) aliquots were added to each well. Thereafter the microplates were incubated for 4h at 37°C and the MTT-formazan crystals formed were dissolved through addition of 100 µl/well 5% formic acid solution in 2-propanol. The MTT-formazan absorption was measured using Beckman-Coulter DTX800 multimode microplate reader at 580 nm. Cell survival fractions were calculated as percentage of the solvent-treated control. In addition, IC_{50} values were derived from the concentration-response curves, using non-linear regression analysis (Curve fit, GraphPad Prizm software).

Apoptotic DNA fragmentation assay (Cell death detection™ ELISA)

The apoptotic DNA fragmentation was examined using a commercially available 'Cell-death detection' ELISA kit (Roche Applied Science). The assay allows semi-quantitative determination of the characteristic for the apoptotic process histone-associated DNA-fragments using 'sandwich' ELISA. In brief, treated or control exponentially growing cells were exposed to varying concentrations of the tested compounds and thereafter cytosolic fractions of 1×10^4 cells per group (treated or control) served as antigen source in a sandwich ELISA, utilizing primary anti-histone antibody-coated microplate and a secondary peroxidase-conjugated anti-DNA antibody. The photometric immunoassay for histone-associated DNA fragments was executed according to the manufacturers' instructions at 405 nm, using Beckman-Coulter DTX800 multimode microplate reader. The results are expressed as the oligonucleosomal enrichment factor (representing a ratio between the absorption in the treated vs. the solvent-treated control samples).

Flow-cytometric analysis of cell cycle progression

The cell cycle progression of KG-1 cells treated with sub-proapoptotic levels of hyperatomarin was assessed by flow-cytometric analysis (FCM) as described elsewhere [54]. In brief, control or treated cells were pelleted, washed with cold PBS, and resuspended in a mixture of 100 µl PBS and 300 µl 96% ethanol. The cells were kept at –20°C. Before the FCM measurements the cells were centrifuged and resuspended in 500 µl PBS, containing 20 µg/ml RNAase and 20 µg/ml propidium iodide (PI) at room temperature. The test tubes were incubated at 4°C for 1 hour, protected from light, and the red fluorescence emitted from the PI–DNA complex was analyzed after laser excitation of the fluorescent dye at 488 nm by FACS Canto II flow cytometer (B-D). DNA QC particles (B-D) and FACS Diva (B-D) were used to set instrument photomultiplier tube voltages and amplifier gains, check instrument resolution and linearity, and verify instrument alignment. At least 20 000 events were collected for each sample at a resolution of 262 144 linear channels using linear amplification of all signals. Thereafter cell cycle distribution was examined by flow cytometric analysis. The percentage of cell distribution data for each treatment group shown is the mean of three independent experiments. The different populations were defined on histograms and expressed as percentages, by means of ModFit LT ver 3.0 software.

Angiogenesis assay

The angiostatic potential of hyperatomarin was evaluated in human umbilical vein endothelial cells (HUVECs), using the MTT-assay and 'Cell death detection' ELISA as bioassay end-points. HUVECs were seeded either to 50% confluence, a point of cell growth mimicking an endothelial cell undergoing angiogenic proliferation. HUVECs were prestimulated with 10 ng/ml vascular endothelial growth factor (VEGF) for 24 h. This exposure intensity proved to be optimal for angiogenic stimulation in a preliminary experiment; data not shown). Thereafter HUVECs were treated with hyperatomarin or with the reference antiangiogenic compound thalidomide for 48h. Then the cellular viability was assessed by the MTT-dye reduction assay and the proportion of apoptotic cells was determined by the 'Cell death detection ELISA' kit (See above).

Data processing and statistics

The MTT-bioassay data are representative for eight independent experiments. The DNA-fragmentation and flow cytometry studies were run in triplicate. The cell survival data were normalized as percentage of the solvent-treated control (set as 100% viability), were fitted to sigmoidal dose response curves and the corresponding IC_{50} values (concentrations causing 50% suppression of cellular viability) were calculated

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Table 1. Antiproliferative effects of hyperatomarin against human tumor cell lines and VEGF-stimulated semi-confluent HUVECs, after 48 h treatment. The equieffective IC_{50} concentrations were calculated from the experimental bioassay data using non-linear regression analysis (GraphPad Prizm software).

Cell line	*Cell type/Origin*	*IC_{50} (μM)*		
		Hyperatomarin	**Daunorubicin**	**Thalidomide**
KG-1	Acute myeloid leukemia (AML)	1.97± 0.11	0.92 ± 0.06	n.d.
HL-60	AML	2.15 ± 0.08	1.1 ± 0.06	n.d.
HL-60/Dox	AML, MDR-resistant variant	1.79 ± 0.81	> 10	n.d.
5637	Urinary bladder cancer	1.19 ± 0.11	2.07 ± 0.11	n.d.
MDA-MB-231	Breast cancer	0.86 ± 0.04	2.11 ± 0.47	n.d.
HUVECs	Umbilical vein endothelial cells	6.09 ± 2.20	n.d.	119.3 ± 6.14

by non-linear regression analysis, using the following equation: $Y=100/(1+10^{((LogIC50-X)*HS))}$; where X is the logarithm of concentration; Y is the response (cell survival); HS is the Hill slope factor (GraphPad Prizm Software for PC) [55].

The statistical processing of biological data included the Student's t-test whereby values of $p \leq 0.05$ were considered as statistically significant.

Results

The antiproliferative/cytotoxic properties of hyperatomarin were evaluated in a spectrum of human tumor cell lines, representative for some important types of leukemia and solid tumors, after 48h treatment, using the MTT-dye reduction assay. The bioassay data were normalized as percentage of the solvent-treated controls and fitted to sigmoidal dose-response curves, using non-linear regression analysis. The corresponding IC_{50} values are summarized in **Table 1**. The clinically utilized anthracycline antibiotic daunorubicin was exploited as reference anticancer agent.

The tested prenylated acylphloroglucinol exerted profound cytotoxic activity causing half-maximal suppression of cellular proliferation at very low micromolar concentrations. The obtained IC_{50} values, were actually comparable or even lower to those of the reference antineoplastic agent. The most eminent response was recorded in MDA-MB-231 and 5637 cells, whereas the leukemic cells were generally inhibited at somewhat higher concentrations.

The emergence of pleiotropic drug resistance, due to overexpression of drug-efflux transporters from the ATP-binding cassette family is a significant hurdle, limiting the usefulness of diverse classes of natural antineoplastic drugs. Therefore we aimed at comparing the activity of hyperatomarin in the MRP-I overexpressing cell line HL-60/Dox versus the corresponding chemosensitive variant. As evident from the results obtained, while daunorubicin was ineffective in HL-60/Dox, hyperatomarin not only bypassed the resistance mechanism but even exerted superior activity in the resistant than in the sensitive cell line.

The ability of hyperatomarin to induce apoptosis was assessed after 24 h exposure to different doses of the compound in HL-60 and KG-1 cells, using a commercially available 'Cell death detection' ELISA kit. As the apoptotic process is typically associated with cascade recruitment and activation of the caspase enzymes, which represent a family of specific proteases, we aimed at investigating the level of DNA-fragmentation following co-incubation of the cell lines with the non-selective pan-caspase inhibitor Boc-Asp(OMe)-fluoromethyl ketone (PCI).

The 24 h treatment of both cell lines with hyperatomarin alone led to a strong, statistically significant increase in the level of DNA fragments. These findings unambiguously indicate that the antiproliferative effects of hyperatomarin, are at least partly mediated by induction of cell death through apoptosis. The concomitant treatment of cells with the phloroglucinol and PCI led to a drastic reduction in the levels of histone-associated DNA-fragments. These data point out that hyperatomarin-induced apoptosis proceeds via activation of the caspase signaling cascade.

To further elucidate the mechanistic aspects of the antiproliferative effects of hyperatomarin in cancer cells, we investigated its effect on cell cycle progression in KG-1 leukemic cells. Consistent with its antiproliferative effects hyperatomarin treatment was consistent with a significant ($P < 0.05$) G1 arrest in exposed cells (**Figure 2**). When applied at the lower dose of 0.15 μM for 24 h hyperatomarin caused an accumulation of ca. 55% cells in G1 phase as compared with the solvent-treated control showing 33.5%. Maximal G1 arrest was observed at the higher level of 0.3 μM whereby the percentage of the G1 population raised more than two-fold to reach approximately 70%. The established increase in G1 cell population was concomitant with a concentration-dependent decline in the percentage of cells in both S phase as well as G2 phase (**Figure 3**).

The angiostatic potential of hyperatomarin was tested in an *in vitro* angiogenesis assay, using VEGF-stimulated primary HUVECs, seeded at 50% confluence mimicking the situation of the active revascularization process. Evident from the results summarized in **Table 1** the tested phloroglucinol proved to

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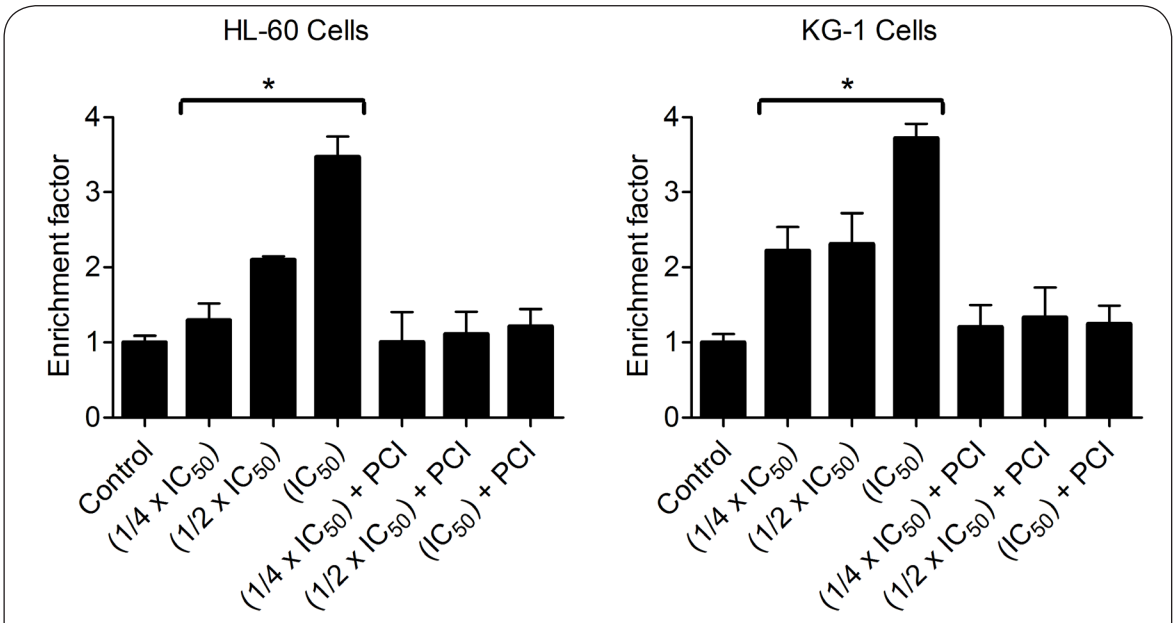

Figure 2. Induction of apoptosis by hyperatomarin treatment in KG-1 and HL-60 cells. Cells were treated for 24 h with different concentrations of hyperatomarin with and without a pan-caspase inhibitor (PCI). Apoptosis was determined by the 'Cell Death Detection ELISA™' kit as described in "Materials & Methods." Each bar is representative for the results of three independent experiments shown as means ± SD. *indicates statistical significance, $P < 0.05$.

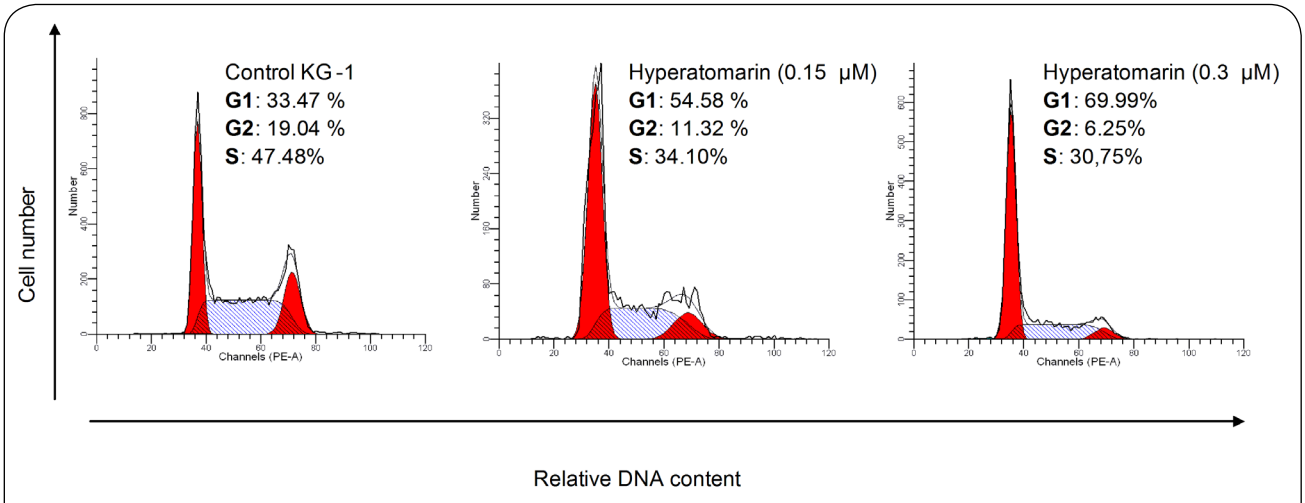

Figure 3. Effects of hyperatomarin on the cell cycle progression in KG-1 cells. Exponentially growing cells were cultured in RPMI 1640 supplemented with 10% FBS, and treated with either vehicle or sub-proapoptotic concentrations of hyperatomarin (0.15 or 0.3 µM). After 12 h exposure, cells were collected, washed with PBS, digested with RNase and then cellular DNA was stained with propidium iodide as described in 'Materials and methods'. Thereafter cell cycle distribution was examined by flow cytometric analysis. The percentage of cell distribution data for each treatment group shown is the mean of three independent experiments.

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be a potent inhibitor of the HUVECs proliferation, causing 50% inhibition of endothelial cells growth at approximately 20 times lower concentration as compared to the reference antiangiogenic agent thalidomide. A DNA-fragmentation study revealed that when applied at 10 μM for 24 h hyperatomarin caused a significant, over two-fold increase ($p \leq 0.01$) of the levels of histone- associated mono- and oligonucleosomal DNA fragments (enrichment factor 2.41 ± 0.12 %), as compared to the solvent-treated control (enrichment factor 1.00 ± 0.17 %), which implies the involvement of apoptotic mechanisms in the antiangiogenic activity of the phloroglucinol.

Discussion

In continuation of our ongoing program for identification and pharmacological exploration of antineoplastic compounds from Hypericum species abundant in the Bulgarian Flora [39,40,56] we hereby report a detailed study on the cytotoxic, proapoptotic and angiostatic activities of hyperatomarin, a prenylated phloroglucinol from *H. annulatum* Moris *subsp. annulatum*.

Hyperatomarin was evaluated for cytotoxicity in a panel of chemosensitive tumor models and one multi-drug resistant (MDR) cell line. It caused strong, concentration-dependent suppression of malignant cell growth, in corroboration to a preliminary pharmacological study [39]. More importantly a prominent collateral sensitivity phenomenon was encountered in HL-60/Dox (a cell line characterized with over expression of MRP-1), *i.e.* the resistant cell line was more responsive as compared to the chemosensitive parent line HL-60. MDR is a phenomenon, whereby cancer cells exposed to one anticancer drug acquire resistance to various agents that are both chemically and pharmacologically distinct from the initially utilized medicine [57-60]. Although there are several alternative mechanisms implicated in MDR [57,61,62], this phenomenon is typically associated with overexpression of the ATP-binding cassette (ABC) family of membrane transporters, including MRP-1, capable of pumping anticancer drugs out of cells, thus lowering their availability at the intracellular target-sites to levels devoid of cytotoxic activity [57,63,64]. Hence the emergence of drug resistance in tumors that have been initially treatment-responsive is a major hurdles limiting the usefulness of antineoplastic chemotherapy [65], there has been a profound interest towards agents with selective cytotoxicity against the emerging MDR-positive cells, as one major approach towards bypassing this resistance mechanism [63,65,66].

As the induction of programmed cell death is of paramount importance for the selective inhibition of cancer cells we sought to determine the proapoptotic activity of the phloroglucinol after treatment with a range of concentrations for 24h. Hyperatomarin caused strong increase in cellular levels of histone-associated DNA-fragments, in a concentration-dependent manner. These findings firmly indicate that the induction of apoptosis plays central role in the cytotoxicity mode of action of this natural compound, which corroborates the data from its pilot pharmacological study [39]. Moreover proapoptotic activity has been encountered with other acylphloroglucinols as well [10,36,40,41,67], which implies that the recruitment of the programmed cell death signaling pathways is responsible for the cytotoxicity of acylphloroglucinols as a class of cytotoxic agents. When the cellular treatment was performed in combination with a pan-caspase inhibitor the levels of DNA-fragmentation were similar to those in the control samples. These findings suggest the possible involvement of caspases activation as one of the potential mechanisms of apoptosis induction by hyperatomarin, whose intimate mechanistic aspect however are yet to be determined. The most prominent acylphloroglucinol hyperforin has been also shown to trigger apoptosis via activation of the caspase-dependent cell death signaling pathways [36,67].

Based on the growth inhibitory and pro-apoptotic effects of hyperatomarin, we further examined its effect on cell cycle progression at concentrations that were devoid of apoptotic activity, as evidenced by the absence of sub-G1/G0 population (apoptotic cells). The 12 h treatment with the phloroglucinol evoked a G1 arrest in KG-1 cells, accompanied with a concomitant reduction in the percentage of G2 and S-phase populations. The G1 cell cycle arrest can prevent the replication of damaged DNA and, therefore, is helpful in checking the uncontrolled proliferation of cancer cells [68,69]. On these grounds the modulation in the cell cycle progression could at least partly contribute to the antiproliferative effects of hyperatomarin.

In order to elucidate the angiostatic potential of hyperatomarin we tested its ability to inhibit VEGF-induced proliferation of HUVECs. The growth factor-stimulated abnormal proliferative response is a crucial event involved in tumor-induced neovascularization and hence this is a widely used screening model to assess the antiangiogenic activity of drugs or drug candidates [70-72]. Hyperatomarin evoked strong inhibitory activity, far more pronounced than that of the clinically used antiangiogenic agent thalidomide. This promising activity prompted us to elucidate mechanistically the observed antiproliferation, using a commercially available 'Cell death detection ELISA™" kit. The established oligonucleosomal fragmentation of the DNA in treated HUVECs is indicative for a proapoptotic mechanism. These findings support the large body of evidence for the antiangiogenic potential of acylphloroglucinols from Hypericaceae species in different *in vitro* and *in vivo* test systems [33,38,43,44,48,49]. Moreover in corroboration to our findings it has been well established that the antiangiogenic effects of the structurally related agent hyperforin are also mediated by induction of apoptosis in endothelial cells [10,38,43].

Conclusions

The main findings of the present study indicate that

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hyperatomarin is a potent cytotoxic and proapoptotic agent, against chemosensitive and multidrug-resistant tumor cell lines, and human endothelial cells stimulated to proliferate by VEGF. Hyperatomarin treatment was found to induce G1 arrest and to induce apoptotic DNA-fragmentation in leukemic cells, presumably via activation of the caspase signaling cascade. Our data indicate that hyperatomarin has multimodal activity targeting tumor cells and abnormally proliferating endothelial cells. On these grounds hyperatomarin could be considered a promising drug candidate with hybrid cytotoxic and angiostatic properties necessitating further detailed pharmacological evaluation as an antineoplastic agent.

Abbreviations

ABC - ATP-binding cassette proteins;
HUVECs – human umbilical vein endothelial cells;
MDR - multidrug resistance;
MRP-1 – multidrug resistance-associated protein;
MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide;
VEGF – vascular endothelial growth factor.

Author information

[1]Department of Pharmacology and Drug Toxicology, Faculty of Pharmacy, Medical University - Plovdiv, Bulgaria.
[4]Department of Pharmacognosy, Faculty of Pharmacy, Medical University-Sofia, Bulgaria.
[5]Section of Botany and Pharmacognosy, Department of Pharmaceutical Sciences, Faculty of Pharmacy, Medical University - Plovdiv, Bulgaria.
[6]Department of Pharmaceutical Technology and Biopharmaceutics, Faculty of Pharmacy, Medical University - Sofia, Bulgaria.
[7]Laboratory of Hematopathology and Immunology, National Specialized Hospital for Active Treatment of Hematological Diseases, Sofia, Bulgaria.
[8]Department of Immunology, National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

PN, DJ-D and GK were involved in the plant collection and identification, isolation and purification of hyperatomarin. DM prepared all drug solutions and participated in the evaluation of the cytotoxic and antiangiogenic properties of hyperatomarin. SB was involved in all pharmacological investigations, data processing and statistics. GM coordinated the pharmacological studies and drafted the manuscript. NS and AM performed the flow-cytometric studies under the supervision of MG. MK coordinated the project and helped compile the discussion of the results. All authors read and approved the final manuscript.

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