STUDIES ON CYTOTOXIC EFFECTS OF 4-HYDROXYCOUMARIN DERIVATIVES IN HUMAN TUMOR CELL LINES

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ABSTRACT

Fifteen 4-hydroxycoumarin derivatives were synthesized and were described. By means of X-ray crystal structure analysis of 4-hydroxy-3-[(2-oxo-2H-chromen-3-yl)-(2-ethoxycarbonylphenyl) methyl]-chromen-2-one (1), 4-(bis[4-hydroxy-2-oxo-2H-chromen-3-yl)methyl]benzoic acid (3) and 3-[2,6-dichlorophenyl] (ethoxy) methyl]-4-hydroxy-2H-chromen-2-one (6) were confirmed the structure of these compounds previously by us. It was investigated the cytotoxicity of 15 4-hydroxycoumarin derivatives. The experiments are conducted on suspension cell lines from human origin- SKW-3 (T-cell chronic lymphocyte leukemia), REH (B-cell precursor leukemia), HL-60 (acute human myeloid leukemia) and K-562 (chronic human myeloid leukemia).

The results of the MTT-test show that the compounds have concentration-dependent antiproliferative activity. The highest average activity observed for compounds 3-[6-oxo-(6H,7H)-benzopyran-4,3-b]benzopyran-10-hydroxy-7-yl]-4-hydroxy-2H-chromen-2- on (13) and 3-[6-oxo-(6H,7H)-benzopyran-4,3-b]benzopyran-9-brom-7-yl]-4-hydroxy-2H-chromen-2-on (14).

Due to its biochemical properties coumarin is of great interest. In particular way, its physiologic, bacteriostatic and antitumor properties make it attractive for screening and derivation as new therapeutic agents. Weber et al. [1] have proven that coumarin and its metabolite 7-hydroxycoumarin have antitumor activity in several human tumor cell lines. Coumarin and coumarin-related compounds have shown promising potential as inhibitors of cell proliferation in different carcinoma cell lines [2-4]. In addition, 4-hydroxycoumarin and 7-hydroxycoumarin have inhibited cell proliferation in stomach cancer cell lines [5].

Results and discussion

Results of MTT-test

Antineoplastic activity of 4-hydroxycoumarin derivatives was determined in cell lines SKW-3, REH, HL-60 and K-562 through MTT-method. Cells were treated for 72 hours with concentrations in the interval of 12.5 - 500 µM.

1. Study on effects of 4-hydroxycoumarin derivatives on SKW-3 cell line

The most cytotoxic effect on SKW-3 cell line showed 3-[6-oxo-(6H,7H)-benzopyran-4,3-b]benzopyran-9-brom-7-yl]-4-hydroxy-2H-chromen-2-on (14). (IC₅₀= 4,308 µM for 72 hours). This compound is with the most strongly expressed cytotoxicity of all the investigated agents in our study. This cell line is the most sensual one against the coumarin treatment with IC₅₀ rate under 70 µM in over 50% of compounds, too. This compound has formula C₂₅H₁₀BrO₆ molar mass 409 g/mol. In its structure has two benzo-α-pyrene rings, one of them, substituted in 4-position with hydroxyl group, and the other ring- substituted in 5-position with bromine atom. 4-Hydroxy-3-[2-oxo-2H-chromen-3-yl)]-(3-methoxy-4-hydroxyphenyl)methyl]chromen-2-on (8), 3-[6-oxo-(6H,7H)-benzo-pyran-4,3-b]benzopyran-8-brom-10-benzoxylo-7-yl]-4-hydroxy-2H-chromen-2-on (9), 3-[6-oxo-
(6H,7H)-benzopyran[4,3-b]benzopyran-8-bromo-11-acetoxy-7-yl]-4-hydroxy-2H-chromen-2-on (10) and 3-[6-oxo-(6H,7H)-benzopyrano[4,3-b]benzopyran-10-hydroxy-7-yl]-4-hydroxy-2H-chromen-2-on (13) showed good antineoplastic effects, too. This derivatives have IC\(_{50}\) respectively 16.92 μM, 20.27 μM, 22.96 μM, 14.73 μM for 72 hours. SKW-3 is the most resistant cell line against the effect of 4-hydroxy-3-[(2-oxo-2H-chromen-3-yl)-(4-carboxyphenyl)methyl]-chromen-2-on (3), (IC\(_{50}\)= 168.2 μM for 72 hours). There are presented comparative graphs, which show survival of SKW-3 cell line after its treatment with all investigated substances.

Graphs 1 and 2: Survival of SKW-3 cells after their treatment with substances 1, 2, 3, 4, 5 and 6.
Graphs 3, 4, 5: Survival of SKW-3 cells after their treatment with substances 7, 8, 9, 10, 11, 12, 13, 14 and 15
2. Study on effects of 4-hydroxycoumarin derivatives on HL-60 cell line

The strongest cytotoxicity on HL-60 cell line showed 3-[6-oxo-(6H,7H)-benzopyran[4,3-b]benzopyran-10-hydroxy-7-yl]-4-hydroxy-2H-chromen-2-on (13), (IC_{50} = 7,514 μM for 72 hours).

A good antineoplastic effect also could be seen from 3-[2,6-dichlorophenyl] (etoxy)methyl]-4-hydroxy-2H-chromen-2-on (6), 4-hydroxy-3-[2-oxo-2H-chromen-3-yl]-(3-methoxy-4-hydroxyphenyl)methyl]-chromen-2-on (8), 4-hydroxy-3-[2-oxo-2H-chromen-3-yl]-(2,3,6-threebromo-5-methoxy-4-hydroxyphenyl)methyl]-chromen-2-on (11) and 3-[6-oxo-(6H,7H)-benzopyran[4,3-b]benzopyran-9-brom-7-yl]-4-hydroxy-2H-chromen-2-on (14), which have IC_{50} respectively 27,85 μM, 44,16 μM, 44,48 μM, 26,88 μM for 72 hours.

It was proven HL-60 is most resistant against the action of 4-hydroxy-3-[(2-oxo-2H-chromen-3-yl)]-(2-benzensulfonyl-3-methoxy-6-nitrophenyl)methyl]-chromen-2-on (15), (IC_{50} = 462,1 μM for 72 hours).

Below are presented succeesively comparative graphs, which show survival of HL-60 cells after the treatment with all investigated substances.

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Graphs 6 and 7: Survival of HL-60 cells after their treatment with substances 1, 2, 3, 4, 5 and 6
Graphs 8, 9 and 10: Survival of HL-60 cells after their treatment with substances 7, 8, 9, 10, 11, 12, 13, 14 and 15
3. Study on effects of 4-hydroxycoumarin derivatives on REH cell line

The strongest cytotoxicity on REH cell line showed 4-hydroxy-3-[(2-oxo-2H-chromen-3-yl)-(2,3,6-threebrom-5-methoxy-4-hydroxyphenyl)methyl]-chromen-2-on (11), (IC<sub>50</sub> = 17.81 μM for 72 hours).

A good antineoplastic effect could be seen from 4-hydroxy-3-[(2-oxo-2H-chromen-3-yl)-(2-etoxycarbonylphenyl)methyl]-chromen-2-on (1), 3-[2,6-dichlorphenyl](etoxy)-methyl]-4-hydroxy-2H-chromen-2-on (6), 4-hydroxy-3-[(2-oxo-2H-chromen-3-yl)-(3-methoxy-4-hydroxyphenyl)methyl]-chromen-2-on (8), 3-[6-oxo-(6H,7H)-benzopyran-4,3-b]benzopyran-8-brom-10-benzoyloxy-7-yl]-4-hydroxy-2H-chromen-2-on (9) and 3-[6-oxo-(6H,7H)-benzopyran-4,3-b]benzopyran-10-hydroxy-7-yl]-4-hydroxy-2H-chromen-2-on (13), which have IC<sub>50</sub> respectively 60.23 μM, 59.40 μM, 31.93 μM, 29.45 μM, 55.90 μM for 72 hours. It was proven REH is most resistant against the action of 4-hydroxy-3-[(2-oxo-2H-chromen-3-yl)-(3-bromo-4-hydroxyphenyl)methyl]-chromen-2-on (5), (IC<sub>50</sub> = 250.7 μM for 72 hours).

Below are presented successively comparative graphs, which show survival of REH cells after the treatment with all investigated substances.
Graphs 12 and 13: Survival of REH cells after their treatment with substances 4, 5, 6, 7, 8 and 9

Graphs 14 and 15: Survival of REH cells after their treatment with substances 10, 11, 12, 13, 14 and 15
4. Study on effects of 4-hydroxycoumarin derivatives on K-562 cell line

The most cytotoxic effect on K-562 cell line showed 3-[6-oxo-(6H,7H)-benzopyrano[4,3-b]benzopyran-10-hydroxy-7-yl]-4-hydroxy-2H-chromen-2-on (13), (IC$_{50}$= 36,76 μM for 72 hours).

A good antineoplastic effect we can see from 4-hydroxy-3-[(2-oxo-2H-chromen-3-yl)-(3-brom-4-hydroxyphenyl)methyl]-chromen-2-on (5), 3-[2,6-dichlorophenyl](ethoxy)methyl]-4-hydroxy-2H-chromen-2-on (6), 4-hydroxy-3-[(2-oxo-2H-chromen-3-yl)-(2,3,6-threebrom-5-methoxy-4-hydroxyphenyl)methyl]chromen-2-on) (11) and 3-[6-oxo-(6H,7H)-benzopyrano[4,3-b]benzopyran-9-brom-7-yl]-4-hydroxy-2H-chromen-2-on (14), which have IC$_{50}$ respectively 74,95 μM, 74,76 μM, 49,21 μM, 74,89 μM for 72 hours. It turned out that K-562 cell line is most resistant to the action of 3-[6-oxo-(6H,7H)-benzopyrano[4,3-b]benzopyran-8-nitro-11-methoxy-7-yl]-4-hydroxy-2H-chromen-2-on (12), (IC$_{50}$= 272,1 μM for 72 hours).

Below are shown successively comparative graphs, which show survival of K-562 cells after the treatment with all investigated substances.
Graphs 17, 18, 19: Survival of K-562 cells after their treatment with substances 4, 5, 6, 7, 8, 9, 10, 11 and 12

Graph 20: Survival of K-562 cells after their treatment with substances 13, 14, 15

Table 4: Cell’s vitality after the
Influence of 3-[6-oxo-(6H,7H)-benzopyran[4,3-b]benzopyran-9-brom-7-y]l]-4-hydroxy-2H-chromen-2- on (14) on the expression of bcl-xl and proteins, connected with the activation of apoptosis

Pharmacodynamic studies were focused on 3-[6-oxo-(6H,7H)-benzopyran[4,3-b]benzopyran-9-brom-7-y]l]-4-hydroxy-2H-chromen-2-on (14), that showed the most promising activity within the screening study for cytotoxicity. It was held immunoblot after SDS-PAA electrophoresis of whole cell lysates from cell lines with CLL and AML with purpose to study the influence of (14) on the expression of different apoptosis factors. SKW-3 and HL-60 cells were treated with (14) on concentrations between 2.25 - 27 µM. The received results were compared with the untreated controls and with the values that are received for β-Actin. Its level is constant and does not change under the influence of the applied cytostatics. All used concentrations were compliant with the average inhibitory concentrations for the substances of the corresponding cell lines. It was investigated the expression of caspase-8, caspase-9 and bcl-xl. It was reported and the level of β-Actin (45 kDa), used as reference. This protein is expressed constitutionally in the cells and it is a part of the cytoskeleton of each cell. Upon induction of cell death its level is kept comparatively constant and it falls to the so-called "house-keeping genes", which can be used as reference by densitometric determination of the target proteins. The analysis of the data showed a reduction of the precursor of caspase-8 in SKW-3 cells after the treatment with 2.25 and 4.45 µM of (14). The test compound also suppresses the expression of bcl-xl. It has not been observed fragmentation of caspase-9 in SKW-3 cell line.

4-hydroxycoumarin derivatives application

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<th>SKW-3</th>
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Experimental part

Chemistry

Melting points were measured on Boetius hot plate microscope (Germany) and are uncorrected. IR spectra (nujol) were recorded on an IR-spectrometer FTIR-8101M Shimadzu. 1NMR spectra were recorded at ambient temperature on a Bruker 250 WM (250 MHz) spectrometer in [d<sub>6</sub>]-acetone, CDCl<sub>3</sub>. Chemical shifts are given in ppm (δ) relative to TMS used as an internal standard. Mass spectra were recorded on a Jeol JMS D 300 double focusing mass spectrometer coupled to a JMA 2000 data system. The compounds were introduced by direct inlet probe, heated from 50 °C to 400 °C at a rate of 100 °C min-1. The ionization current was 300 mA, the accelerating voltage 3 kV and the chamber temperature 150 °C. TLC was performed on precoated plates Kieselgel 60 F254 (Merck, Germany) with layer thickness of 0.25 mm and UV detection (254 nm). Yields of TLC-homogeneous isolated products are presented. Results of elemental analyses were within ± 0.4 % of the theoretical values.

General Procedure

4-Hydroxycoumarin and the respective aromatic aldehyde at a molar ratio 2:1 in glacial acetic acid were mixed under stirring and heated at reflux until the appearance of an insoluble product. After cooling the product was filtered and was recrystallized. The following eight 3,3’-arylidenebis-(4-hydroxy-2H-1-benzopyran-2-ones), six benzopyrano-coumarins and a 2,6-dichlorophenyl-ethoxymethyl-4-hydroxycoumarin derivatives were synthesized according to this procedure.
4-Hydroxy-3-[(2-oxo-2H-chromen-3-yl)-(2-ethoxy carbonylphenyl)methyl]-chromen-2-one (1)
4-Hydroxycoumarin, 2-formylybenzoic acid, 144 h, ethanol (6 h, gl. acetic acid), recrystallized from acetonitril, 63 %, 228-231 °C [7-9].

4-Hydroxy-3-[(2-oxo-2H-chromen-3-yl)-(3-carboxyphenyl)methyl]-chromen-2-one (2)
4-Hydroxycoumarin, 3-formylybenzoic acid, 6 h, gl. acetic acid, recrystallized from ethanol, 67 %, 228-231 °C [7].

4-Hydroxy-3-[(2-oxo-2H-chromen-3-yl)-(4-hydroxyphenyl)methyl]-chromen-2-one (3)
4-Hydroxycoumarin, 4-formylybenzoic acid, 16 h, ethanol, 72%, 280-282 °C. [7, 8, 10, 11, 12]

4-Hydroxy-3-[(2-oxo-2H-chromen-3-yl)-(4-hydroxyphenyl)methyl]-chromen-2-one (4)
4-Hydroxycoumarin, 4-hydroxybenzaldehyde, 7 h, acetic acid, recrystallized from ethanol, 69 %, 212-214 °C [7, 13 - 23].

4-Hydroxy-3-[(2-oxo-2H-chromen-3-yl)-(3-bromo-4-hydroxyphenyl)methyl]-chromen-2-one (5)
4-Hydroxycoumarin, 3-bromo-4-hydroxybenzaldehyde, 9 h, recrystallized from ethanol, 80 %, 182.3-184 °C [7].

3-[2,6-Dichlorophenyl](ethoxy)methyl]-4-hydroxy-2H-chromen-2-one (6)
4-Hydroxycoumarin,2,6-dichlorobenzaldehyde, 13 h, ethanol, 78 %, 132-133 °C [7, 24].

3-[6-oxo-(6H,7H)benzopyrano[4,3-b]benzopyran-8,10-dimethoxy-7-yl]-4-hydroxy-2H-chromen-2-one (7)
4-Hydroxycoumarin, 4,6-dimethoxy-2-hydroxybenzaldehyde, 16 h, gl. acetic acid, 62 %, 245.2-247 °C [7, 25, 26].

4-Hydroxy-3-[(2-oxo-2H-chromen-3-yl)-(3-methoxy-4-hydroxyphenyl)methyl]-chromen-2-one (8)
4-Hydroxycoumarin, 3-methoxy-4-hydroxybenzaldehyde 11 h, gl. acetic acid (recrystallized from ethanol), 70 %, 215-216.4 °C [7, 27 - 30].

3-[6-oxo-(6H,7H)benzopyrano[4,3-b]benzopyran-8-bromo-10-benzoyloxy-7-yl]-4-hydroxy-2H-chromen-2-one (9)
4-Hydroxycoumarin, 4-benzoyloxy-6-bromo-2-hydroxybenzaldehyde, gl. acetic acid, 21 h, recrystallized from ethanol, 74 %, 252-254.3 °C [7].

3-[6-oxo-(6H,7H)benzopyrano[4,3-b]benzopyran-8-bromo-11-acetoxy-7-yl]-4-hydroxy-2H-chromen-2-one (10)
4-Hydroxycoumarin, 3-acetoxy-6-bromo-2-hydroxybenzaldehyde, gl. acetic acid, 17 h (recrystallized from ethanol), 77 %, 218-220.5 °C [7].

4-Hydroxy-3-[(2-oxo-2H-chromen-3-yl)-(2,3,6-tribromo-5-methoxy-4-hydroxyphenyl)methyl]-chromen-2-one (11)
4-Hydroxycoumarin, 2,3,6-tribromo-5-methoxy-4-hydroxybenzaldehyde, gl. acetic acid, 21 h (recrystallized from ethanol), 59.3 %, 231-233 °C [7].

3-[6-oxo-(6H,7H)benzopyrano[4,3-b]benzopyran-8-nitro-11-methoxy-7-yl]-4-hydroxy-2H-chromen-2-one (12)
4-Hydroxycoumarin, 3-methoxy-6-nitro-2-hydroxybenzaldehyde, gl. acetic acid, 16 h, recrystallized from ethanol, 77.5 %, 217-219 °C [7].

3-[6-oxo-(6H,7H)benzopyrano[4,3-b]benzopyran-10-hydroxy-7-yl]-4-hydroxy-2H-chromen-2-one (13)
4-Hydroxycoumarin, 2,4-dihydroxybenzaldehyde, 13 h, gl. acetic acid (recrystallized from ethanol), 73 %, 267-269.4 °C [7, 14, 15].

3-[6-oxo-(6H,7H)benzopyrano[4,3-b]benzopyran-9-bromo-7-yl]-4-hydroxy-2H-chromen-2-one (14)
4-Hydroxycoumarin, 5-bromo-2-hydroxybenzaldehyde, 13 h, gl. acetic acid (recrystallized from ethanol), 73 %, 307-310 °C (decompn) [7, 25, 31-33].

4-Hydroxy-3-[(2-oxo-2H-chromen-3-yl)-(2-benzensulfonyl-3-methoxy-6-nitrophenyl)methyl]-chromen-2-one (15)
4-Hydroxycoumarin, 2-benzensulfonyl-3-methoxy-6-nitrobenzaldehyde, gl. acetic acid, 32 h (recrystallized from ethanol), 80 %, 152.5-154.5 °C [7].

Used cell lines
The experiments are conducted on cell lines, which are cultivated as suspension cell cultures. They are human cell lines and are obtained as standard cell lines from tumor cell bank DSMZ (Deutsche Sammlung für Mikroorganismus und Zellkulturen – German collection of microorganisms and cell cultures), Braunschweig, Germany.

- SKW-3 – human T- cell leukemia, obtained from peripheral blood of 61-years old patient with T- cell chronic lymphocyte leukemia. Morphologically
are characterized single, round cells, which are growing in suspension. Cytogenetically is defined close to the human diploid karyotype with 4% polyploid cells.

- **REH** – B- cell precursor leukemia, isolated in 1973 from peripheral blood of 15-years old girl from Northern Africa with ALL. Morphologically are characterized round, single cells in suspension. Growing conditions: 37°C, 5% CO₂. Doubling time: 50-70 hours.

- **HL-60** – acute human myeloid leukemia, isolated in 1976 from peripheral blood of 35-years old woman with AML. Morphologically are characterized round, single cells in suspension. Growing conditions: 37°C, 5% CO₂. Doubling time: 40 hours. Cytogenetically they are cells from human hypotetraploid karyotype with hypodiploid side and 1,5% polyploidy.


**Cells treatment with cytostatics**

Cell lines are treated with cytostatics during the period of the exponential phase of growth (log-phase), when the fraction of dividing cells is high (usually 90-100%) and the culture is in its most reproductive period. This period is optimal for treatment, because cells vitality is high.

**MTT-method for assessment of cell vitality**

In 1983 Mosmann describes a colorimetric method which is based on the reduction of tetrazole salts (MTT, HTT) under the action of the mitochondrial enzymes dehydrogenases in live cells. They transform the MTT-dye to insoluble in water violet formazane crystals in quantities straightforward to the number of the viable cells. MTT-formazone is characterized by absorption maximum in the range between 400 и 600 nm length of the wave and the intensity of the samples’ coloring can be measured with spectrometer and calculated in percentage by untreated control that is accepted as 100%. The absorption of the environment and pure solvent is measured parallel in advance left well as referent solution (blank sample) and the value is subtracted from the one of the samples.

**Fig. 6: Reduction of tetrazole salt MTT to formazone**

The MTT-method was used from us for the evaluation of cell lines vitality, treated with different cytostatic substances alone or in combination.

**Gene expression assay**

In the framework of this paper was investigated the expression of different cell proteins, which take part in transduction signaling pathways of apoptosis and in cell cycle’s regulation. The extent of expression of these signaling molecules was determined at the protein level with the help of Western blot.

**Determination of the protein concentration by the method of Pierce (BSA Protein Assay)**

In protein expression analysis, cell lysates, cytoplasmic and nuclear fractions, made from each sample, are fractionated with polyacrylamide gel electrophoresis. The protein concentration in each sample is determinate before protein’s separation in the gel as the fractionation of equal amount of total protein is a necessary condition for comparing the intensity of the protein strips after the visualization.
"Pierce BSA Protein Assay" is a highly sensitive method for quick and accurate spectrophotometric determination of protein concentration. It has been developed on the basis of famous biuret reaction for protein’s proving by reduction of cupri- (Cu²⁺) to cupro- ions (Cu⁺⁺) in alkaline environment. Bicynchonic acid (BCA) is a highly sensitive and selective reagent for discovering of Cu⁺⁺-ions. When determining with BCA in the samples with macromolecule structures or the peptides cystein, cystin, tryptophan and tyrosine, is forming purple reaction product. Its formation is a result of the interaction of two molecules BCA with one copper (II) ion (Cu²⁺). The resulting compound is soluble in water and has absorption maximum between 540 and 600 nm:

**Immunoblot**

The immunoblot is an analysis, based on the reaction antigen-antibody. In this analysis, protein molecules are transferred via transverse electrophoresis, under the action of an electric field, from the gel on a membrane, adhered to its large part. Transverse electrophoresis was conducted at so-called “semi-dry method”. The detection of the searching proteins is based on the reaction antigen-antibody, that is going on the surface of the membrane. It is necessary pre-blocking of the free places on its surface to reduce the opportunity for non-specific binding of the antibodies. For the detection of the searching protein was used indirect method and antibodies, conjugated with horseradish peroxidase.

**CONCLUSIONS**

It is investigated the cytotoxicity of 15 4-hydroxycoumarin derivatives. The experiments are conducted on suspension cell lines from human origin- SKW-3 (T-cell chronic lymphocyte leukemia), REH (B-cell precursor leukemia), HL-60 (acute human myeloid leukemia) and K-562 (chronic human myeloid leukemia).

The results of the MTT-test show that the compounds have concentration-dependent antiproliferative activity. The highest average activity have compounds (13) and (14). The most pronounced cytotoxicity is reported in SKW-3 cell line with substance (14) (IC₅₀ = 4,308 µM for 72 hours). To study the influence of (14) on the expression of different signal molecules, related with the regulation of apoptosis, was performed an immunoblot after SDS-PAAG electrophoresis of whole cellular lysates from cell lines with CLL and AML- origin. The datas analysis showed reduction of the precursor of caspase-8 in SKW-3 cells after treatment with 2,25 and 4,45 µM (14), but it is not observed fragmentation of caspase-9. The test compound suppresses the expression of bcl-xl, too.

The test compound activates the external, receptor signal way of the programmed cell death but not the internal, mitochondrial way. Suppression of the expression of antiapoptotic regulatory protein bcl-xl at least in part mediates the cytotoxic effects.
Submitted data outlines (14) as a perspective leadership structure with expressed cytotoxic activity, that is mediated by activation of the apoptogenic signal ways and modulation of the antiapoptotic bcl-xl.

REFERENCES


