

Thesis for the degree of Doctor of Philosophy (Ph.D.)

Experimental data for therapy of human uveal melanoma

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DEPARTMENT OF OPHTHALMOLOGY
MEDICAL AND HEALTH SCIENCES CENTER
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**Experimental data for therapy of human uveal
melanoma**

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1. INTRODUCTION AND LITERARY SURVEY

1.1. General description of intraocular tumors

An intraocular tumor is the mass, setting out from the eye-ball tissues and it infiltrates and damages its surroundings. It takes up the place of normal tissues to larger or smaller extent, and sometimes fills up the bulbus partially or fully.

In organogenetical respect we can differentiate between benignus (non-malignant) and malignus (malignant) ocular tumors.

The types of ocular malignant tumors in their frequent occurrence: melanoma, malignum uveae, retinoblastoma, metastatic tumors, lymphoma, medulloepithelioma.

Among them, uveal melanoma has the biggest practical importance, as it is the only one potentially fatal ocular malignant tumor.

Uveal melanoma is a scarce disease, comprising 2.9 % of all melanomas and less than 1% of all registered tumors. It is a highly malignant intraocular tumor, with bad prognosis and currently unknown aetiology. It can be of primary and secondary occurrence. It is usually unilateral but we know its bilateral presence, too, even in the form of metastasis.

It is a relatively scarce tumor, however, this is the second frequent human primary melanoma. The death rate of the uveal melanoma is high, due to the haematogenic spreading.

As there are no lacteals inside the eye-ball, metastasis occurs almost only in haematogenic way. The liver is the main 'target organ' of metastases.

About the incidence of intraocular tumors including uveal melanomas: Studying statistics of recent years - there are no reliable data available. Some say that it has been increasing lately, others say that it hasn't. Making correct statistics is difficult as the ocular and intraocular tumors are not registered precisely and not distinguished, and the primary and metastatic tumors are not separated even in the ICD (International Classification Of Diseases) code.

According to surveys made in the USA, uveal melanomas are 19.4 times frequent in white adults than in black adults, and the rate of men is higher in all ethnographic groups. It can occur in any age-group, but the peak of the incidence is between the 40-60th years of life.

Four congenital uveal melanomas have been described in the literature till now. Its childhood incidence is really scarce, only 18 cases have been described among children under 16 years old till now.

In Hungary we have 25-30 new cases a year, most of them are treated at the Ophthalmic Clinic of Debrecen University of Medicine.

Regarding its clinical importance and use, the classification based on the anatomical position of tumors is really important. Melanoma malignum uveae can refer to any tissues of the uveal tract, it can set out from any part of it: it can be found in the iris, the corpus ciliare and the chorioidea, too. Their frequent occurrence is: iris 6%; corpus ciliare 9% chorioidea 85%.

Melanomas with clinically similar appearance can show very different tissue types, from the low-malignant fuscellular A type that rarely gives distant metastasis, to the fuscellular B type and the high-malignant, often metastatic epithelioid cellulous melanomas.

An attention-drawing connection has been found between the location of the tumor and the prognosis. Iris melanomas have the best prognosis, and the corpus ciliare tumors have the worst one.

Due to the anatomical localisation it is the easiest to identify iris melanomas, even in case of small-sized ones. So their diagnosis is relatively easy. Their mortality is 3-5%/10 years. Metastasis was found in 3% in 5 years, in 5% in 10 years and in 20% in 20years. The prognosis of corpus ciliare melanomas is worse. After enucleatio the 5-year mortality is 53%, while after the enucleatio of the chorioidea melanoma the rate of metastasis was 14%.

The average time of metastasis development is 68 months. The development of metastasis is supposed if the biggest prominentia of the tumor is more than 7mm. Although the average size of the corpus ciliare melanomas is already bigger at the diagnose than the size of those in the chorioidea, statistics showed that the prognosis in these cases is not dependent on the size of the tumor or the cell-type.

Heading towards the rear pole, the frequent occurrence of tumors and malignancy of the melanoma is increasing: it is the least frequent in the iris and the most frequent in the chorioidea. Tumors in the chorioidea close to the macula lutea are the most malignant.

There can be uveal melanoma and naevus in one eye at the same time.

The 5-year mortality of small tumors (whose diameter is less than 7 mm, prominentia is less than 2 mm) is less than 4%. Bigger tumors show metastasis in 50% in 3 years.

1.2. Inhibition of proliferation

As the uveal melanoma is highly resistant to the currently used chemotherapeutic medicines, there are no efficient chemotherapeutic medicines by which the uveal melanoma can be cured.

The development of the successful *immune therapy* is largely delayed by the fact, that the eye— to a certain extent- is in immunologically privileged position, as both the acquired and the inherited immune responses are suppressed.

Moreover, the effects of the ocular microenvironment largely influence the reactions of the uveal melanoma cells inhibiting lymphocyte-functions.

As a consequence, *it is urgent to find new ways of treating uveal melanomas.*

The discovery of antigens connected to uveal melanomas – both *in vivo* tumorous cells and *in vitro* on melanoma cell lines – refers to the fact that in certain circumstances these tumors can have immunological points of attacks and this fact can make these tumors the effective targets of dendritic-cell based immune-therapies.

Not long ago it was pointed out that if apoptotic melanoma cells were given to dendritic-typed cells, they were able to produce proliferative and cytotoxic T-cell response. This suggests that the dendritic cells - produced this way - *in vivo* were able to increase the effectiveness of apoptosis inducing

antitumor medicines and *in vitro* enables it to create effector T-cells for adaptational transfer therapy.

That's why it is not simply important but even of key-importance to examine new compounds that can have antiproliferative effects on melanoma cells. In the future they – developed into medicine – can compliment the dendritic based therapeutic protocols or the long-applied, well tried brachytherapeutic treatment.

Recently the analysis of medicines of plant origin in clinical researches has been highlighted in order to prevent the development of tumors and also to treat already existing ones. These compounds have become more well-spread for the present time in therapies of treating tumors.

It had already been proved that alkaloids isolated from *Chelidonium majus* and other *Papaveraceae* plant family had a wide range of biological activity from antimicrobial effects to anti-inflammatory effects. We can group the alkaloids of the *Chelidonium majus* according to their chemical structure. We can distinguish protopine-typed (e.g. a- and b-allocryptopine, protopine), protoberberine-typed (e.g. berberine) and benzophenanthridine-typed (e.g. chelidonine, sanguinarine, chelerythrine, chelilutine, chelirubine, macarpine) alkaloids. The anti-inflammation and anti-tumor effects of the latest have already been proved. Among them chelidonine, sanguinarine (in other words pseudocheleytrine) and chelerythrine were profoundly analysed.

The three compounds mentioned above were proved to be inhibitory in cell-growing through inducing apoptosis. This suggests that they can be potentially used as proapoptotic medicines in tumor-therapy. Moreover, these compounds proved to be effective against tumors that had been resistant to the already existing standard therapies.

Though nowadays we have just few reliable data on the effects of chelidonine, that is the main component of the *Chelidonium majus*, but these facts indicate that this benzophenanthridine-typed alkaloid can also induce apoptosis-typed cell death in altered inner signaled and malinus cells. The chelidonine inhibits the microtubulus polymerization ($IC_{50}=24 \mu M$), by this it tears apart the microtubulus structure of the cells.

Sanguinarine – beside its several effects – inhibits the Na⁺/K⁺-ATP-ase , the protein kinase A and the activation of NF-kappa B. Chelerythrine inhibits the protein kinase C and increases the release of cytochrome-C, which has an important role in the apoptosis induction in continuing the process.

These compounds have not been tested against the uveal cell-line before, that's why we became interested in their application.

Beside the already existing treatments of uveal melanoma we should find new ones.

One future possibility can be the completion of brachytherapy – that has been used many times at the Department of Ophthalmology of University of Debrecen – with suitable anti-tumor agents. These agents can sensitize the tumor cells towards radiotherapy and can increase the effects of brachytherapy.

Numerous compounds with supposed antitumor activity have been tested on uveal melanoma cells. It has been proved during the analysis of the biological activity of oligonucleotides containing thio-deoxyuridylate that though the short oligomers are inactive, the monomer – which substance occurs in nature, too – induces apoptosis in tumor cells.

Though tumor-research has developed a lot in the recent years, decades, and the circle of therapies have been increasing as well, we do not have a chance in the treatment of melanoma malignum chorioideae by which *in vivo* we could intervene in the growth of tumors at cellular level.

That's why our work has been focused on the analysis of the possibilities of the antiproliferative treatment. If we can realize achievements during laboratory work, and apply them on the living organisation we could be successful in the more effective treatment of melanoma patients.

2. OBJECTS

1. Our main objective was the analysis of the possible antiproliferative treatments of melanoma malignum uveae, by which we can intervene in the growth of tumors at cellular level.
2. Our aim was to choose such a cell-line that shows quite aggressive proliferation, that is available in great quantities and very characteristic of the intraocular physiological behaviour of uveal melanomas. We wanted to influence the lifeperiod of the cell-line at several points of effect, with several substances.
3. We wanted to be the first to study the effect of benzophenanthridine alkaloids on uveal melanoma cells. If the benzophenanthridine alkaloids are able to inhibit cell-proliferation successfully, we wanted to clear up whether the cells had died by apoptosis or necrosis. So we studied the effects of chelerythrine, chelidonine and sanguinarine among benzofenanthridine alkloid compounds.
4. To inhibit OCM-1 uveal melanoma cells we also used a nucleotide that can be found in nature, 4-thio-uridylylate. First, we wanted to examine whether this nucleotide provides significant reduce in cell-proliferation. If yes, we wanted to clear it up which mechanism it can be obtained by.

3. MATERIALS AND METHODS

3.1. OCM-1 cell culture

For our analysis we wanted to choose a cell-line that shows quite aggressive proliferation, that is available in great quantities and very characteristic of the intraocular physiological behaviour of uveal melanomas. That's why we chose the OCM-1 (ocular choroideal melanoma) cells as they stand out from the other uveal melanomas with their proliferative ability.

For the benzophenanthridine alkaloids researches we put the cells into RPMI (Roswell Park Memorial Institute) 1640 nutritive fluid, which contained 10% FCS {fetal calf serum} 0.3 g/ml L-glutamine and gentamycine as antibiotics, and we incubated it in steamy substance containing 5% CO₂ at 37 °C. We passed the cells twice or three times a week using standard trypsin method.

Using with the 4-thio-uridyate, the RPMI contained FBS (phosphate buffered saline) inactivated by 10% heat and as antibiotics it contained penicillin in 100 international unit/ml dose and streptomycin in 100 µ/ml dose. In the latter the storage of the cells happened at 37 °C, in steamy substance containing 5% CO₂.

After the incubation we filtered the cells through a mixture containing PBS/trypsin with 2.5µg/ml concentration.

3.2. Benzophenanthridine alkaloids

3.2.1. Benzophenanthridine alkaloid compounds

From among benzophenanthridine alkaloids we used chelerythrine, chelidonine and sanguinarine, which we get from the Sigma Aldrich Co.

The chelidonine was dissolved in dimethyl-sulphoxide, and the chelerythrine-chloride and the sanguinarine-chloride was dissolved in DMSO/water (1:2) agent.

3.2.2. Treatment with benzophenanthridine alkaloids

We put the cells into a 24-holed plate. We proportioned about 1×10^5 cells into one hole suspending in 500 μ l solution. We treated the cells – sludging on the wall of the plate in 80-90% -with alkaloids with different concentration: 0.5, 1, 4 and 8 μ g/ml dose.

We went on with the treatment until the 4th, 24th and 48th hours. Taking into consideration the mostly identical molar mass of the analysed alkaloids, it is calculable that the applied doses meant roughly identical molar concentration.

The solutions were diluted with the suitable solvent every time in order to have the same final DMSO (dimethyl-sulphoxide) concentration.

The control-cells were treated with the same amount of DMSO and kept under the same experimental circumstances. During the time mentioned above, the cells were corroded by trypsin, washed by PBS (phosphate-buffered saline) and prepared for DNA fragmentation assay or annexin V/PI assay. Under the effects of the alkaloid treatments, the debris came off the wall of the plate and floated in the solution.

3.2.2.1. DNA fragmentation assay

During the analysis connected to benzophenanthridine alkaloids, we determined the DNA content of the cells by flow cytometry. The cells were centrifugated at 200 g, then kept in 0.5 hypotonic fluorochrome solution (50 μ g/ml PI (propidium-iodide) in 0.1 % Triton X 100) before flow cytometry.

The apoptotic cells were recognisable as their DNA concentration was smaller, e.g. the characteristic sub- G_1 peak on the DNA-content (PI-intensity) frequency histogram.

During the analysis concerning s^4 UMP we treated 2×10^6 cells with s^4 UMP for 24 and 48 hours, then we collected the cells with centrifugation.

After that we washed them with PBS, then isolated the DNA and put it to agarose gels. The agarose-gels were evaluated by Alphamager™ 2200 and were archived.

3.2.2.2. Annexin V-FITC/PI staining of cells in benzophenanthridine alkaloids' researches

The separation of apoptotic cells from living and necrotic cells took place by using annexin V-FITC (fluorescein isothiocyanate) and PI (propidium-iodod) applying Annexin V-FITC Apoptosis Detection Kit. The centrifugated cells were diluted to 1×10^6 cell/ml concentration by cohesive puffer (10 mM HEPES/NaOH, 0.14 M NaCl, 2.5 mM CaCl_2 , pH=7.5). The samples were incubated in a mixture of 0.5 $\mu\text{g/ml}$ PI at room temperature for 10 minutes and measured by FACScan flow cytometer. We measured the annexin V-FITC and PI fluorescence on FL-1 (green) and FL-2 (red) channels, and evaluated the results after the correction of the spectrum overlappings between the two channels, which we analysed by the help of WinMDI 2.8 or FLEX software.

Separation of apoptotic and necrotic cells was based on annexin V-FITC activity and PI exclusion. The apoptotic cells showed intensive green (FITC) and low or medium red (PI) fluorescence (in accordance with the early or late apoptotic phase). The permeability of cells in the late apoptotic phase changes, this is the consequence of the altered intensity of their plasma membrane. The necrotic cells proved to be stainable from both reagents' respect and this is why they showed strong green and red fluorescence. The living cells were not stained at all.

3.2.2.3. Analysis of cell-morphology

We put the cells on a slide in order to analyse the effects of alkaloids on cell-morphology. A part of them sludged on the slide. We treated those cells with alkaloids which showed 80-90% sludge, and we incubated these for 4 hours, then analysed by Zeiss LSM 510 scanning laser-microscope. We were searching for light-microscopic changes characteristic of apoptosis and/or necrosis.

3.2.2.4. MTT-assay

Execution of the MTT (3-[4,5-dimethyliazol-2-yl]-2,5-diphenyltetrazolium bromid) assay took place according to the instruction for use of the American Type Culture Collection (ATCC). We put the cells into a 48-holed plate, there were 0.6×10^5 per holes in 200 μ l nutritive fluid for 15 hours and we treated them with the alkaloids with labelled doses or without them, just with the solvent.

After 24 hours of incubation, we mixed the cellular-culture with a 200 μ l MTT solution and incubated it for another 3 hours, then we suspended it upwith 200 μ l acid iso-propanol solution and we took 200 μ l of this homogen mixture to a 96-holed plate and read it by ELISA reader.

3.3. Treatment with 4-thio-uridin-5'-monophosphate (s^4 UMP)

3.3.1. Production of 4-thio-uridin-5'-monophosphate s^4 UMP

We produced s^4 UMP thionated mononucleotid by H_2S treatment of citidin-5'-monophosphate and we cleaned it by ionexchanging chromatography, as it was described in earlier publications of our workteam. We prepared 10mg/ml stock-solution and we diluted it in tissue-culture every time immediately before using it.

3.9. Measuring caspase-9 activity

We measured caspase-9 activity in order to clear up the effect-mechanism of s^4 UMP as caspases have a key-role in starting and accomplishing apoptotic events.

s^4 UMP treated OCM-1 cells were centrifugated at 1000 rpm for 10 minutes and washed by PBS twice. We examined activity by Caspase-9/Mch6 Colorimetric Assay Kit in the way described in the instructions for use.

During evaluation we considered the mean value of 3 measurements independent from each other.

3.10. Flow cytometry after s⁴UMP treatment

We incubated both s⁴UMP treated and not treated OCM-1 cells (5x10⁵ cell/hole) for 72 hours and we centrifugated them for 10 minutes at 10 °C. Then we resuspended the cells in 0.5 cohesive puffer (25 mM HEPES, 125 mM NaCl, 2.5 mM CaCl₂), and indicated them with 5 µl annexin FITC and 5 µl propidium iodide according to the instructions for use. (Medical and Biological Laboratories Co. Ltd., Nagoya, Japan). We used FacsCalibur flow cytometer for the flow cytometric measurements of floating, not fixed cells, using 488 nm argon-laser for excitacion. We evaluated the results by CellQuest software. In each case, the fluorescent data collected 20 000 events.

3.11. Statistical analysis

We calculated the statistical facts, the mean and the scattering values by WinMDI 2.8, FLEX, CellQuest and GraphPad PRISM[®] 4 softwares.

4. RESULTS

4.1. Results with benzophenanthridine alkaloids

4.1.1. Divergent effects of chelidonine from sanguinarine and chelerythrine

Benzophenanthridine alkaloids have already been proved to inhibit the growth of tumor-cells in different types of tumors. In order to prove whether they effect OCM-1 uveal melanoma cells in the same antiproliferative way, we did colorimetric MTT-assay. The cell viability of OCM-1 cells showed strong and dose-dependent decrease after 24-hour sanguinarine and chelerythrine treatment.

However, the proliferation-decrease caused by chelidonine was hardly considerable and its effect in all of the analysed concentration-domains (0.5 µg/ml-8 µg/ml) was not significantly dose-dependent. Concerning chelidonine, the antiproliferative effect, different from the other two alkaloids, has already been described regarding other cell-types.

4.1.2. Morphological divergencies on OCM-1 cells under the effect of benzophenanthridine alkaloids

Although just a low cell-growing inhibition has been proved concerning chelidonine, we experimented cell-morphological changes by light-microscopic analysis: it indicated apoptosis in OCM-1 cells even after 4 hours.

In the samples that contained only vehicles, cells stuck on the wall of the plate, while the chelidodnine –treated cells came off the plate-wall, and many of them showed the blebbing of the plasma membrane, that is one of the strong alterations, characteristic of apoptosis.

Light-microscopic analysis showed dose-dependent alterations in sanguarine and chelerytine cases. Cells treated with a large dose (8 µg/ml) of sanguarine and chelerytine showed stronger cell-swelling, which alteration can

be seen in the early phase of necrosis. When we decreased the concentration of the effective agent, the number of necrotic cells decreased as well, and at the same time cell-morphological alterations, characteristic of apoptosis appeared, too.

In the lowest concentration (0.5 µg/ml) most cells had a normal shape, similarly to the morphology of the control-cells. From the results mentioned above we can come to the conclusion that sanguarine and chelerytine destroy uveal melanoma cells by two coexisting mechanisms (apoptosis and necrosis).

4.1.3. Quantitative analysis of apoptosis caused by benzophenanthridine alkaloids

We proved by a light-microscopic analysis that benzophenanthridine alkaloids can induce apoptosis in OCM-1 uveal melanoma cells. We applied two analysing methods in order to measure quantitatively the apoptosis-inducing ability of these alkaloids, by which we could demodulate alterations, characteristic of apoptotic cells.

4.1.3.1. DNA fragmentation assay

One characteristic of apoptosis is the appearance of fragmented DNA with small molecular-weight. These can be shown by flow-cytometry. So first we evaluated the apoptosis-inducing effect of alkaloids by their DNA fragmentation quality. After 4-hour treatment neither chelidonine, nor sanguarine caused significant DNA degradation, but after 24-hour incubation the proportion of cells with fragmented DNAs increased. On the contrary, chelerytrin caused significant DNA fragmentation even after 4-hour incubation, and the number of apoptotic cells increased in direct proportion with the longer incubation time.

Concerning chelidonine, the produced reaction was not dose-dependent in the analysed concentration domain. At the same time, alterations under the effect of sanguarine and chelerythrine showed a biphasic character.

4.1.3.2. Analysis of annexin V-FITC/PI staining

We made samples with DNA fragmentation assay in a simultaneous way, which we stained by annexin V-FITC and propidium-iodide, and evaluated by flow-cytometry. The annexin-V binding ability of a cell – linking to phosphatidylserine on the outer surface of the plasma membrane – is another specific sign of apoptosis. Moreover, contrasted to the DNA degradation test if we apply double staining of the cells – annexin-V connected to fluorochrome and a marker analysing plasma membrane intensity (that is propidium iodide) – we can make apoptotic cells visible and also can separate necrotic and living cells from each other.

We have to point it out that – concerning the two assays mentioned above – the absolute number of the apoptotic cells was not necessarily the same. This could happen as applying the two different methods we got different cellular responses or because the preparation, measurement and analyses of the samples occurred in different ways.

We analysed the proportion of the apoptotic cells after 4 and 24 hour incubation with benzophenanthridine alkaloids.

Similarly to the results of the DNA degradation, concerning chelidonine we did not find any significant apoptosis-inducing effects with annexin V-FITC/PI staining. When we increased the incubation time, the number of the apoptotic cells increased significantly, and it also referred to dose-dependency. But we could not demonstrate this dose-dependency by DNA fragmentation assay.

While the previous method could hardly prove it, annexin V-FITC/PI staining of OCM-1 cells well proved the fact that sanguarine caused valuable apoptosis in the analysed concentration-domain after only 4-hour treatment. This result is accordance with the fact that sanguarine induces fast apoptotic response, even in a few hours, due to the great and early glutathione depletion of the cells, then, in the later phases, apoptotic changes are not so dominant.

In our researches, we also experimented the biphasic pattern of sanguarine-induced apoptotic response in accordance with literary facts. While the number of apoptotic cells, analysed by the two quantitative methods,

showed a reversed proportion in time, the two different researches gave similar results for chelerythrine.

In spite of the analysed differences, the two assays obviously demonstrated the apoptosis-inducing effect of benzophenanthridine alkaloids on OCM-1 uveal melanoma cells.

4.1.4. Necrotic cell-death induction in OCM-1 cells under the effect of benzophenanthridine alkaloids

Annexin V-FITC/PI staining proved that the benzophenanthridine alkaloids caused not only apoptosis but also necrosis in OCM-1 cells, depending on the applied doses. This effect characterised chelidonine the least, while in case of sanguinarine and chelerythrine it was bigger. Applying sanguinarine and chelerythrine, even a 4-hour treatment caused significant necrosis, while chelidonine just hardly increased the proportion of the necrotic cells.

The proportion of the necrotic cells was around 10% at the beginning, and under the effect of chelidonine, this proportion increased by only 20-25%.

4.2. The effect of s⁴UMP on OCM-1 cells

4.2.1. MTT-assay

First, we did the MTT-assay in order to analyse the different doses and treatments with different duration on cell-proliferation.

In these experiments we added the substances to the OCM-1 cells just once, in T=0 time. After the 24-hour treatment the cell viability of the cells decreased in all the four concentrations (6, 30, 150 and 300 μ M, which are equivalent to 1, 10, 50 and 100 μ g/ml). There are a lot of explanations of the decrease of the cell viability of the cells, including apoptosis.

4.2.2. Cell morphology

In order to get information of the effect-mechanism of s⁴UMP, first we analysed its effect on nucleotid OCM-1 cell-morphology.

Choosing the 150 µM dose (50 µg/ml) of s⁴UMP nucleotid and the 48 and 72-hour incubation times in the morphology we could see characteristic apoptotic changes and decreased cell-number after the treatment.

4.2.3. DNA fragmentation

One significant sign of apoptosis is the presence of the DNA degradation and the presence of the DNA-ladder with about 180 nucleotid of fragments.

We treated the OCM-1 cells with 30 µM (10 µg/ml) s⁴UMP for 24 and 48 hours, then we isolated the DNA and analysed it with agarose-gel electrophoresis. DNA-degradation was time-dependent, and it proved the characteristic signs of apoptotic degradation.

4.2.4. Caspase-9 activation

For further analysis of the effect-mechanism of s⁴UMP, we determined caspase-9 activity before and after the treatment.

Caspase-9 activity was the highest in 48 hours under the effect of the 90 µM (30 µg/ml) s⁴UMP treatment.

The pattern of the DNA-degradation and the increased caspase-9 activity really suggested that s⁴UMP resulted in apoptosis.

4.2.5. Flow citometry

After that we determined the proportion of annexin-positive and annexin and propidium-iodide cells by FACS analysis before and after the treatment,

too. The results indicated that the main type of s⁴UMP-effect is apoptosis induction and necrosis can appear just secondarily.

5. DISCUSSION

5.1. Effect of benzophenanthridine alkaloids on OCM-1 cells

Summarizing our results of benzophenanthridine alkaloids we can conclude that albeit in different extent, benzophenanthridine alkaloids investigated in this study could induce apoptotic as well as necrotic cell death in OCM-1 uveal melanoma cells. Whereas chelidonine was predominantly effective via apoptosis, sanguinarine and chelerythrine induced a so-termed bimodal cell death (both apoptosis and necrosis).

These results turn our attention to the possible use of chelidonine, sanguinarine and chelerythrine in the treatment of uveal melanomas.

Due to their apoptotic potential these alkaloids are not only good candidates for chemotherapeutic regimens, but may also contribute to the development of successful immune therapies of uveal melanomas. In addition to the promising use of apoptotic uveal melanoma cells in dendritic cell-based immune therapies their application in combination with the expression of costimulatory molecules could provide a novel adjuvant therapy for these tumors.

Analysis of annexin V-FITC/PI staining disclosed, that benzophenanthridine alkaloids of this study also induced a dose-dependent necrosis of OCM-1 cells. This effect was the less pronounced for chelidonine, while sanguinarine and chelerythrine were more efficient. It appears that these remarkable differences in the extent of necrotic response induced by chelidonine *versus* sanguinarine or chelerythrine are account for the differential antiproliferative response of OCM-1 cells for these alkaloids.

Intriguing conclusions could be drawn if we compare the amount of necrotic and apoptotic cells for a given alkaloid. In the case of chelidonine the fraction of apoptotic cells significantly exceeded that of the necrotic ones, implying that under the applied experimental conditions apoptosis is the predominant form of chelidonine-induced cell death.

At the same time it appears that for sanguinarine and chelerythrine the two modes of cell death (apoptosis and necrosis) are competing with each other

and the net effect (the actual ratio of apoptotic and necrotic cells) depends on the concentration, the incubation time and the condition (e.g. confluence) of cells.

As a rule of thumb at higher concentration necrosis is the dominant form of cell death, whereas for lower doses the efficiency of apoptosis could exceed that of necrosis.

These results are in a good accordance with morphological data obtained by light microscopy and explain “biphasic” pattern of the apoptotic response observed for sanguinarine and chelerythrine.

The above findings were also corroborated by analyzing light scattering properties (i.e. size and morphology) of cells by flow cytometry. Changes in the forward and the right angle light scatter signals supported that upon chelidonine treatment cells mainly undergo apoptosis, while sanguinarine and chelerythrine induces bimodal cell death.

Sanguinarine-induced bimodal cell death was reported earlier in several other cell types suggesting that this is a general phenomenon. Similarities between sanguinarine- and chelerythrine-mediated cellular responses presumably arose from the structural homology of these alkaloids.

To our knowledge this is the first study showing the cancer therapeutic potential of chelidonine, sanguinarine and chelerythrine against uveal melanoma cells. However, further studies are required to unravel the exact mechanism(s) of the responses evoked by these alkaloids as well as to verify their effectiveness in a model system. However, the possible side effects should be also investigated.

5.2. Effect of s⁴UMP on OCM-1 cells

Our workgroup has earlier reported the potent *in vitro* anti-HIV activity of a 35-mer oligonucleotide composed exclusively of 4-thiodeoxyuridylates. A detailed study of the mode of action of the molecule and of the effect of the chain length on the antiviral and antiproliferative activities of the oligonucleotides led to the result that the monomer 4-thio-dUMP decreased the viability of several tumor

cell lines, including OCM-1 uveal melanoma cells. This result was rather surprising because we had earlier found that the oligonucleotides composed exclusively of 4-thiothymidylates lost their biological activity at shorter chain length. Ribo- and deoxyribonucleotide (s^4 UMP and s^4 dUMP) decreased the cell viability equally (data not shown); accordingly, all of the experiments were carried out with the ribo derivative s^4 UMP. Since s^4 UMP is a nucleotide, it is not likely to penetrate into the cells; its apoptosis-inducing activity may therefore be due to the interactions between the modified nucleotide and the cell surface proteins. This is chemically feasible because the 4-thiono group has a propensity to undergo tautomeric conversion to form reactive –SH groups at position 4; it can interact with –SH-containing cell surface proteins form disulfide bridges. The reductive function of the cell surface, mediated by surface sulfhydryls, including protein disulfide isomerase, has been described earlier. These –SH-containing proteins are probable targets of s^4 UMP. In line with the above-proposed mode of action, we found that $(s^4dU)_{35}$, a deoxy oligomer of 4-thiolated-uridylylate, could interact with cell surface thioredoxin. Analysis of the effects of s^4 UMP via the MTT assay revealed that its action is dose-dependent, but saturation can be achieved with a high concentration of the drug. When the inhibitor concentration was 6 μ M (2 μ g/ml s^4 UMP), the cell viability was decreased by 20% at 24 h, and a concentration of 300 μ M nucleotide did not prove more active than 150 μ M. The observation that the inhibitory effect can be saturated is in good agreement with the assumed mode of action: the inhibitory nucleotide interacts with the cell surface proteins.

Another important conclusion which could be drawn from the results of the viability assay is that s^4 UMP is metabolized by the cells. Analysis of the effects of 30 μ M (10 μ g/ml) s^4 UMP indicated that the inhibition of cell viability amounted to 32%, 40% and 9% after 24, 48 and 72 h of incubation, respectively. The 9% inhibition at 72 h clearly shows that the cells recovered almost completely after the treatment

6. SUMMARY AND NEW RESULTS

Tumor-research has been improved a lot in the recent years. Diagnostics and therapy of ophthalmologic tumors have also seen a significant development. There are no antiproliferative therapy, however, among our possibilities. During our study we were examining the antiproliferative therapy of uveal melanoma under laboratory conditions. We have managed to reach, under laboratory circumstances, a significant inhibition in the proliferation of OCM-1 uveal melanoma cells.

1. We have managed to acquire for our laboratory work a sort of aggressive tumor, which – compared to other uveal melanoma – had a really significant proliferative capability. OCM-1 uveal melanoma cells proved to be right. We intended to be the first to examine the effect of benzofenantridine alkaloids on human uveal melanoma cells.
2. We have managed to reach significant cell-destruction by first applying benzofenantridine alkaloids in the proliferation-inhibiting process. The decreasing of cell-vitality was justifiable both by biochemic methods and light-microscope. All the three benzofenantridine alkaloids we tried caused both apoptosis and necrosis, but to a different extent. The change compared to control has always justified a significant deviation. Chelidonin first of all caused apoptosis, while sanguinarin and chelerythrin led to – beside apoptosis – necrosis, thus the effect caused by these two compounds can be described as biphasic deviation. The produced effects were dose-related.
3. We have reached positive results in the inhibition of OCM-1 human uveal cells by applying a nucleoid, s^4 UMP. We were the first to examine both the apoptosis induce effect of s^4 UMP, and its effect on OCM-1 uveal cells. It has been verified that this nucleoid, which can be found also in the nature, induce apoptosis in OCM-1 cells.

Further experiments are needed to clear the precise and detailed mechanism, but further on it could be possible to create the antiproliferative effect in the uveal melanoma therapy with the help of the above mentioned compounds or their modified varieties.

7. PUBLICATIONS

7.1. *IN EXTENSO* PUBLICATIONS IN SUPPORT OF THE THESES

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