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Antiproliferative effect of 4-thiouridylate on OCM-1 uveal melanoma cells

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PURPOSE. *Brachytherapy is a well-established, effective treatment for uveal melanoma with a failure rate of 15%. The fatal consequence of unsuccessful treatments offers reason for improvement of the method. The authors propose using an apoptosis inducing agent locally, concomitantly with the well-established therapy, to sensitize the tumor cells. The authors propose a new nontoxic moderately active apoptosis inducing agent, 4-thio-uridylate (s4UMP), for this purpose.*
METHODS. *OCM-1 uveal melanoma cells were treated with various concentrations of s4UMP and its effect was monitored by measuring the cell viability (MTT assay). The following apoptosis detecting methods were performed to reveal the mechanism of decreased cell viability: light microscopy, DNA fragmentation assay, determination of caspase 9 activity, and FACS analysis.*
RESULTS. *The viability of uveal melanoma cells was decreased by 32%, 40%, and 9% after 24, 48, and 72 hours of treatment with 10 µg/mL (30 µM) s^4UMP. The effect was not dose dependent; it rather followed a saturation-type inhibition and the cells at lower drug concentration recovered after 72 hours. Characteristic apoptotic cell morphology and DNA fragmentation was detected in treated cells. The caspase-9 was activated upon treatment showing maximal activity at 48 hours suggesting the induction of apoptosis. The annexin binding activity further verified the apoptogenic activity of s^4UMP.*
CONCLUSIONS. *Uveal melanoma, more than other solid tumors, is resistant to most of the chemotherapeutic protocols as indicated by the high mortality rate of metastatic disease. The authors showed that s^4UMP, a naturally occurring nucleotide, could induce apoptosis in uveal melanoma cells, suggesting a potential supplementary therapeutic application of the compound. (Eur Ophthalmol 2006; 16: 680-5)*

KEY WORDS. *Antitumor, Nucleotide, OCM-1, Uveal melanoma*

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INTRODUCTION

Malignant uveal melanoma is a rare tumor representing less than 1% of cancer registrations; however, it accounts for 13% of deaths from melanoma (1, 2). The failure rate of brachytherapy is about 15%, and resistant tumors are highly metastatic with a poor prognosis (3). More precise understanding of the molecular pathogenesis of uveal melanoma is required for the development of alternative therapies. Recent results, including ours, indicate progress in this field (4-7).

A possible improvement of the treatment could be the combination of brachytherapy with a locally applied antitumor agent. Such agents could sensitize the tumor cells for radiation increasing the effectiveness of the radiation. Several compounds with antitumor ac-

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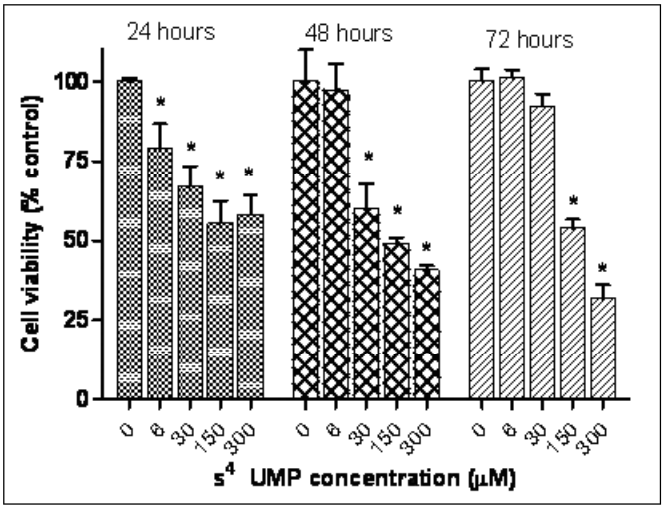

Fig. 1 - *Effects of various concentrations of s^4UMP on viability of OCM-1 cells, as measured with the MTT assay. Values are means and SEM from triplicates. Stars indicate significant difference compared to the control ($p<0.01$).*

tivity were tested on uveal melanoma cell lines with promising activity (8, 9). We found recently that the alkaloids of Chelidonium majus are potent apoptosis inducers in OCM-1 uveal melanoma cells (10).

Studying the biological activity of 4-thio-deoxyuridylate containing oligonucleotides (11), we found that the ribo-monomer (s^4UMP), which is a naturally occurring nucleotide, induces apoptosis in OCM-1 uveal melanoma cells. We describe the results of these experiments.

METHODS

Chemicals and preparation of s^4UMP

Chemicals were purchased from Sigma Aldrich and Amersham Pharmacia and were analytical grade or better. The thiolated mononucleotide, s^4UMP, was prepared by H_2S treatment of Cytidine-5'-monophosphate as we described for deoxy-ribo-oligonucleotides (11) and purified by ion-exchange chromatography (12). A 10 mg/mL aqueous stock solution was prepared from s^4UMP and diluted with tissue culture media before use.

Cell culture

OCM-1 human uveal melanoma cell-line was originally developed by Dr. Kan-Mithell, Department of Microbiology, Norris Cancer Hospital and Research Institute, Los Angeles, California (13). We obtained the cell line from Dr. Monique Hurks, Department of Ophthalmology, Leiden University Medical Center, Leiden, The Netherlands. The cells were cultured in RPMI containing 10% heat-inactivated FBS and antibiotics (100 units/mL penicillin and 100 µg/mL streptomycin), and were grown at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were harvested after incubation with PBS/trypsin (2.5 µg/mL).

MTT assay

To measure the cell viability $5x10^5$ OCM-1 cells were seeded into 24 well-plates. Cells were cultured for 6 hours in 1 mL of medium, as described above, and then treated with various concentrations of s^4UMP. One hundred µL aliquots were removed in triplicate at the indicated time for MTT [3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyl-tetrazolium bromide] assay. The assay was performed as described (14) and specified by the manual of American Type Culture Collection (ATCC).

DNA fragmentation analysis

Two x 106 cells were treated with s^4UMP for 24 and 48 hours as indicated, collected by centrifugation (1000 rpm, Jouan C/CR4-12, Horizontal Rotor; 10 min, 10 °C), washed twice with PBS, then DNA was isolated and subjected to agarose gel-electrophoresis (15). The agarose gels were evaluated and archived by AlphaImager™ 2200.

Measurement of caspase-9 activity

OCM-1 cells treated with s^4UMP were centrifuged at 1000 rpm for 10 min and washed twice with PBS. Detection of the caspase-9 activity was achieved using a Caspase-9/Mch6 Colorimetric Assay Kit, according to the instruction of manufacturer (Medical and Biological Laboratories Co., Ltd., Nagoya, Japan). The results represent the average of three independent experiments.

Flow cytometry

OCM-1 cells were cultured for up to 72 hours in the presence or absence of s^4UMP. Cells ($5x10^5$ cells/tube) were centrifuged (1000 rpm, Jouan C/CR4-12, Horizontal Rotor) for 10 min at 10 °C then resuspended in 0.5 mL binding buffer (25 mM HEPES, 125 mM NaCl, 2.5 mM $CaCl_2$) and were labeled by 5 µL annexin-FITC and 5 µL propidium iodide according to the instruction of manufacturer (Medical and Biological Laboratories Co., Ltd.). Flow cytometric

measurements were carried out on unfixed cells by a FacsCalibur flow cytometer (Becton Dickinson, Biosciences, San Jose, CA) using a 488 nm argon laser excitation. Data were stored in list mode files and were analyzed by CellQuest software. In all cases fluorescence data of 20,000 events were collected.

Statistical analysis

Data were expressed as means and SEM calculated by GraphPad PRISM® 4 software.

RESULTS

First we studied the effect of doses and the lengths of treatment on the cell proliferation by MTT assay (Fig. 1). In this experiment the drug was added to the OCM-1 cell-culture at zero time only once. At 24 hours of treatment the cell viability is decreased at all of the four concentrations applied (6, 30, 150, and 300 μM, equivalent to 2, 10, 50, and 100 μg/mL).

The decreased cell viability may be accounted for by various processes including apoptosis. To get some information on the mode of action of s^4UMP we first studied the effect of the nucleotide on the morphology of OCM-1 cells. A series of experiments was completed using the same protocols for treatments as shown in Figure 1. We present here the change of morphology after 48 and 72 hours of treatment with 150 μM s^4UMP. A characteristic apoptotic change in morphology and decreased cell number was observed upon the treatment with the modified nucleotide (Fig. 2).

A hallmark of apoptosis is the degradation of DNA and the formation of a characteristic DNA ladder with about 180-nucleotide increments. We treated OCM-1 cells with 30 μM (10 μg/mL) s^4UMP and isolated DNA after 24 and 48 hours of treatment then analyzed it by agarose gel electrophoresis. The DNA fragmentation was time dependent showing the characteristic pattern of apoptotic degradation (Fig. 3).

To analyze further the mode of action of s^4UMP the caspase-9 activity was determined before and after the treatment (Fig. 4). The caspase-9 activity was the highest at 48 hours of treatment with 90 μM (30 μg/mL) s4UMP. The pattern of DNA degradation and the elevated caspase-9 activity strongly suggests that s^4UMP induces apoptosis.

Then we determined the ratio of annexin positive

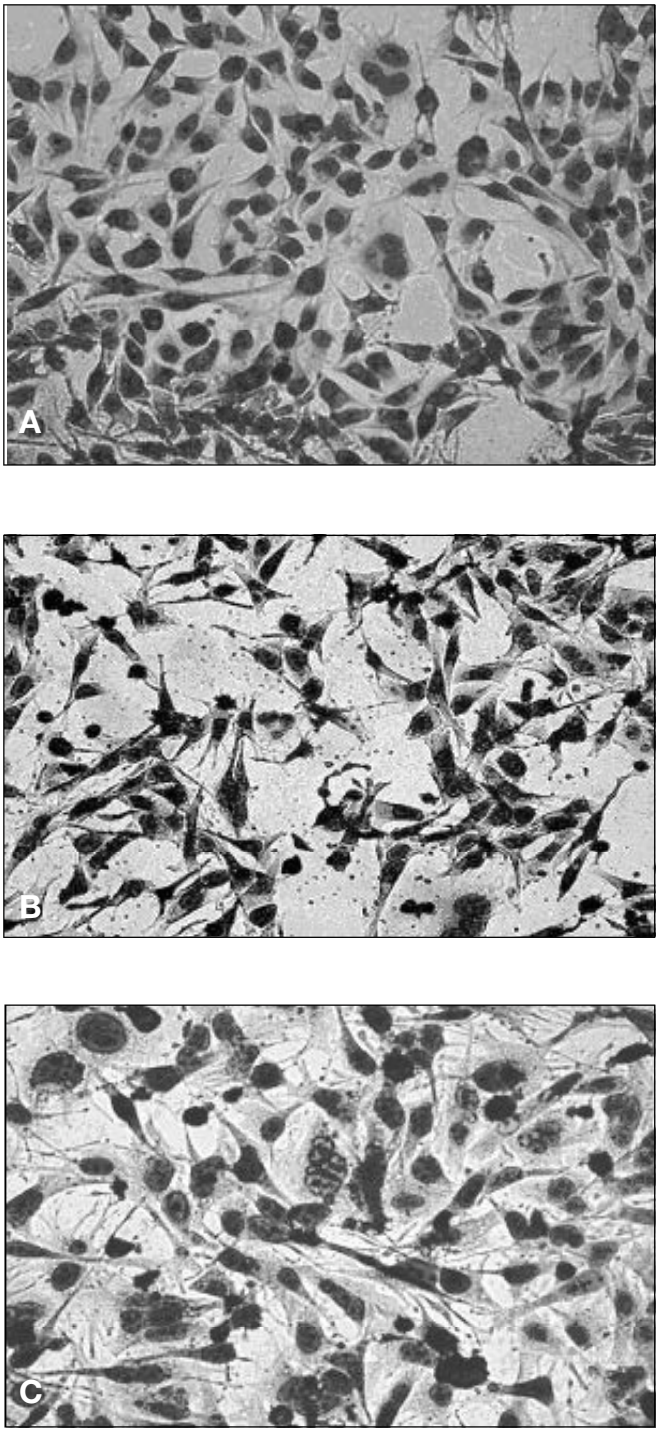

Fig. 2 - *Effects of* s^4UMP *on morphology of OCM-1 cells. Cells were stained with May-Grünwald-Giemsa and evaluated with the use of a Zeiss Axivert 135 microscope. Magnification 200x.*
A) *Control cells after 48 h of propagation;* **B**) *OCM-1 cells treated for 48 h with 150 μM (50 μg/mL)* s^4*UMP;* **C**) *OCM-1 cells treated for 72 h with 150 μM (50 μg/mL)* s^4*UMP.*

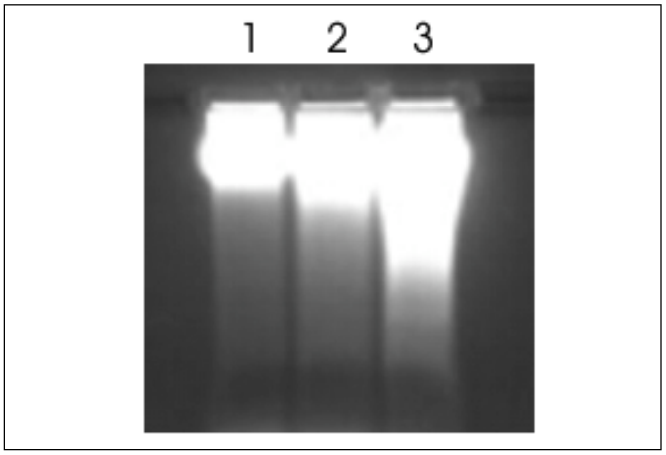

Fig. 3 - *DNA fragmentation of OCM-1 cells, induced by 30 µM (10 µg/ml) s4UMP.* (**1**) *Control;* (**2**) *after 24 h of treatment;* (**3**) *after 48 h of treatment.*

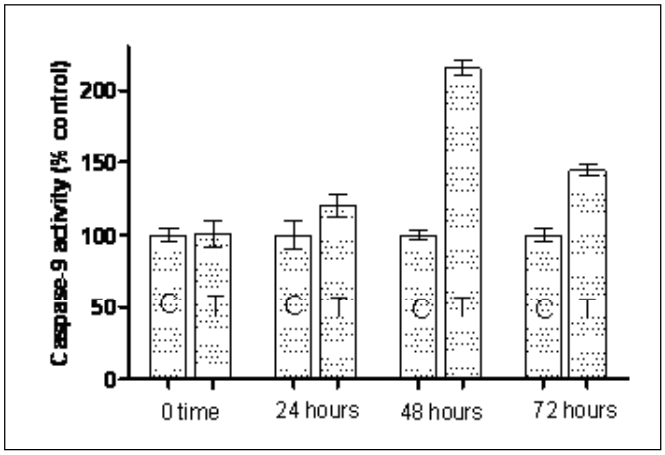

Fig. 4 - *Activation of caspase-9 upon treatment with 90 µM (30 µg/ml) s4UMP (C: control; T: treated). Values are means and SEM from triplicates.*

and annexin and propidium iodide positive cells before and after treatment by FACS analysis. The results (Tab. I) indicate again that the main mode of action of s^4UMP is induction of apoptosis and only secondary necrosis may appear.

DISCUSSION

We reported earlier the potent in vitro anti-HIV activity of a 35-mer oligonucleotide composed exclusively of 4-thio-deoxyuridylates (11). A detailed study, thoroughly examining the mode of action of the molecule and the effect of chain length on the antiviral and antiproliferative activity of the oligonucleotides, has 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 surprising because we found earlier that the oligonucleotides, composed of 4-thio-deoxyuridylates exclusively, were losing their biological activity at shorter chain length. Both ribo- and deoxyribonucleotide (s^4UMP, s4dUMP) decreased the cell viability equally (data not shown); therefore all of the experiments were completed with the ribo-derivative: s^4UMP (4-thio-uridine 5'-phosphate).

Since s^4UMP is a nucleotide, it is not likely to penetrate into the cells; therefore, its apoptosis inducing activity may be due to the interactions between the modified nucleotide and cell surface proteins. This is chemically feasible because the 4-thiono group has a propensity toward tautomeric conversion (16) to form reactive –SH groups at position 4, thus, it can interact with –SH containing cell surface proteins forming disulfide bridges. The reductive function of cell surface, mediated by surface sulfhydryls including protein disulfide isomerase, has been described earlier (17, 18). These –SH containing proteins are likely targets of s^4UMP. We found that $(s^4dU)_{35}$, a deoxy-oligomer of 4-thiolated-uridylate, could interact with cell sur-

TABLE I - CHANGE IN PROPORTIONS OF ANNEXIN-POSITIVE AND ANNEXIN-PROPIDIUM IODIDE (ANNEXIN-PI)-POSITIVE CELLS ON TREATMENT WITH s^4UMP

Duration of treatment (h)	Untreated		Treated	
	Annexin-positive	Annexin-PI-positive	Annexin-positive	Annexin-PI-positive
0	33	6		33 7
24	21	8	41	8
48	15	6	46	15

Values are percentages

face thioredoxin (11) in line with the above proposed mode of action. Further studies are required to elucidate the exact mode of action of s^4UMP and find those proteins (if any) which interact with the thiolated nucleotide. Such a study is beyond the scope of this article.

Analyzing the effect of s^4UMP by MTT assay we found that its effect is dose dependent but can be saturated with high concentration of drug (Fig. 1). When the inhibitor concentration was 6 μM (2 μg/mL s^4UMP) the cell-viability decreased by 20% at 24 hours and 300 μM nucleotide was not more active than 150 μM drug (Fig. 1, at 24 hours). The observation that the inhibitory effect can be saturated is in good agreement with the assumed mode of action: the inhibitory nucleotide is interacting with cell surface proteins.

Another important conclusion which could be drawn from the results of the viability assay is that s^4UMP is metabolized by the cells. Analyzing the effect of 30 μM (10 μg/mL) s^4UMP we found that the inhibition of cell-viability was 32%, 40%, and 9% after 24, 48, and 72 hours of incubation. The 9% inhibition at 72 hours indicates that the cells recovered almost completely after the treatment (Fig. 1).

In a preliminary experiment we have studied the in vivo toxicity of s^4UMP. It proved to be nontoxic in mice (our unpublished result). Since we found that the compound can be metabolized and the cells were recovered after longer period of incubation the two seemingly contradictory observations, the apoptosis inducing activity and the nontoxic nature of s^4UMP, could be understood.

The DNA degradation, the caspase-9 activation, and the data of FACS analysis are consistent with an apoptotic process, although the exact pathway is not known.

This is the first publication on the apoptosis inducing activity of s^4UMP. A highly resistant and aggressive tumor model was chosen for this study, the OCM-1 uveal melanoma cells. The apoptosis inducing feature of s^4UMP suggests that it is a potential antitumor agent and may have utility in the treatment of malignancies perhaps as a nontoxic supplementary therapeutic agent sensitizing the tumors for apoptosis.

The study of apoptosis inducing activity of s^4UMP and structurally related compounds on a number of tumor cell lines with diverse origin is in progress in our laboratory.

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Dr. Janos Aradi is the patenter of the antiproliferative activity of 4-thio-uridilate and related compounds. However, the owner of right is University of Debrecen.

Proprietary interest: none.

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