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Induction of γ-globin mRNA, erythroid differentiation and apoptosis in UVA-irradiated human erythroid cells in the presence of furocumarin derivatives

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ABSTRACT

Psoralens, also known as furocoumarins, are a class of photosensitizers largely used in the therapy of various skin diseases. In this study we have evaluated the combined effects of UVA irradiation and furocoumarins derivatives on (a) erythroid differentiation and apoptosis of human leukemia K562 cells and (b) globin gene expression in cultures of human erythroid progenitors derived from the peripheral blood. To prove the activity of a series of linear and angular furocoumarins derivatives, we employed the human leukemia K562 cell line and the two-phase liquid culture procedure for growing erythroid progenitors. Quantitative real-time reverse transcription polymerase-chain assay (Q-RT-PCR) was employed for quantification of the accumulation of globin mRNAs. The results obtained demonstrate that both linear and angular furocoumarins are strong inducers of erythroid differentiation of K562 cells. From a preliminary screening, we have selected two derivatives, 5-methoxypsoralen (5-MOP) and trimethylangelicin (TMA), for which we have investigated their mechanism of action. The cell cycle analysis showed that these derivatives induce, after irradiation, a cell cycle arrest in the G2/M phase, followed by apoptosis. Mitochondrial depolarisation and caspases activation seem to be involved in the mechanism of cell death. In erythroid precursor cells, psoralens in combination with UVA irradiation, stimulate at very low concentrations a preferential increase of γ-globin mRNA. Altogether, these data suggest that psoralen derivatives warrant further evaluation as potential therapeutic drugs in β-thalassemia and sickle cell anemia.

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Abbreviations: 5-MOP, 5-methoxypsoralen; 8-MOP, 8-methoxypsoralen; $\Delta\psi_{mt}$, mitochondrial potential; ANG, angelicin; BFUe, erythroid burst forming units; CFUe, erythroid colony forming units; EPO, erythropoietin; HBSS, Hank's balanced salt solution; HE, hydroethidine; ICL, interstrand cross-link; NAO, 10-*N*-nonyl-acridine orange; PI, propidium iodide; PUVA, psoralen plus UVA; ROS, reactive oxygen species; TMA, trimethylangelicin.

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1. Introduction

The search for potential therapeutic agents in haematological diseases, including β-thalassemia and sickle cell anemia (SCA), focuses on the pharmacologically mediated regulation of the expression of human γ-globin genes [1–5]. In fact, an increase as low as 30% of production of fetal hemoglobin (HbF, $\alpha_2\gamma_2$) leads to a significant improvement of the clinical status of the patients [1]. Therefore, many studies have started to find natural or synthetic compounds capable of augmenting HbF levels in humans. In particular, emphasis has been given to DNA-binding agents, which appear of great interest [6–8].

Psoralens, also known as furocoumarins, are naturally occurring or synthetic tricyclic aromatic compounds deriving from the condensation of a coumarin nucleus with a furan ring. Their planar structure helps them to intercalate between nucleic acid base pairs. UVA irradiation activates the intercalated complex, resulting in the formation of photoadducts with pyrimidine bases in cellular DNA [9]. The psoralen monoadducts formed in the DNA can further react photochemically with a pyrimidine base on the complementary strand of the DNA, thus leading to interstrand cross-links (ICL), believed to be the primary cause of photoinduced cell killing [10]. New psoralen derivatives have been synthesized, such as the angular psoralen angelicin. It allows only monofunctional photobinding, thus reducing undesirable side effects, especially long-term ones, such as genotoxicity and risk of skin cancer [11].

Many human skin diseases, such as psoriasis, T-cell lymphoma (cutaneous T-cell lymphoma, CTCL), and vitiligo are commonly treated with a combination of psoralens and UVA radiation commonly referred to as PUVA (psoralen plus UVA) therapy.

Furthermore, the combination of psoralen and UVA light is nowadays employed in a modified PUVA protocol called extracorporeal photopheresis (ECP). This treatment consists in the exposition of leukopheresed concentrated white blood cells to 8-methoxypsoralen administered directly into the white blood cell bag and UVA light and then returned to the patient. This method is currently used for treating CTCL [12], and other T-cell-mediated diseases, such as organ transplantation and graft versus host disease [13–15].

Since the accumulation of γ-globin mRNA in human erythroid cell treated with angelicin in the absence of UVA irradiation [16], it is interesting to evaluate the combined effect of psoralen derivatives and UVA light on the induction of erythroid differentiation.

Therefore, the purpose of this study was to determine whether angelicin and other angular and linear furocoumarin derivatives are able to induce erythoid differentiation and to increase the expression of γ-globin genes after UVA irradiation of human erythroid cells.

To verify the activity of several linear and angular derivatives (Schemes 1 and 2) we employed the human erythroleukemia K562 cell line [7] and the two-phase liquid culture of human erythroid progenitors isolated from healthy donors [8]. These experimental approaches are useful in identifying molecules capable of stimulating HbF production in erythroid precursors derived from normal subjects as well as patients with thalassemia and sickle cell anemia [2].

2. Materials and methods

2.1. Chemicals

All the psoralen derivatives used in this study belong to the collection of the Department of Pharmaceutical Sciences, University of Padova. Benzidine, caffeine and 3,4-dihydro-5-[4(1piperidinyil)butoxy]-1(2*H*)-isoquinolinone (DPQ) were purchased from Sigma–Aldrich (Milano, Italy). The pancaspase inhibitor z-VAD.fmk and wortmannin were purchased from Vinci-Biochem (Firenze, Italy).

2.2. Irradiation procedure

Two HPW 125 Philips lamps, mainly emitting at 365 nm, were used for UVA exposure. The spectral irradiance of the source was 4.0 mW cm^{-2} as measured by a Cole-Parmer Instrument Company radiometer (Niles, IL), equipped with a 365-CX sensor.

2.3. Cell cultures and phototoxicity tests

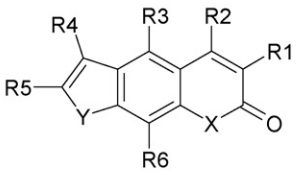
The human leukemia K562 cells were cultured in a humidified atmosphere of 5% CO_2/air in RPMI 1640 medium (Sigma–Aldrich, Milano, Italy), supplemented with 10% fetal bovine serum (FBS; Invitrogen, Milano, Italy), 100 units/ml penicillin and 100 μg/ml streptomycin. Individual wells of a 24-well tissue culture microtiter plate were inoculated with complete medium containing 30,000 of K562 cells per milliliter. The plates were incubated at 37 °C in a humidified 5% incubator for 18 h prior to the experiments. Stock solutions of psoralen derivatives (10 mmol/l) in dimethyl sulphoxide (DMSO) were stored at −20 °C in the dark, diluted immediately before the use with methanol/dimethyl sulphoxide (1:2), and then diluted with Hank's balanced salt solution (HBSS pH 7.2) for irradiation experiments. The concentration of solvent never exceeded 0.5%. After medium removal, 1 ml of the drug solution was added to each well, incubated at 37 °C for 30 min and then irradiated. After irradiation, the solution was replaced with complete medium and the plates were incubated for 5–7 days. The medium was not changed during the induction period. Erythroid differentiation was determined by counting benzidine-positive cells after suspending the cells in a solution containing 0.2% benzidine in 5 mol/l glacial acetic acid, 10% H_2O_2, as described elsewhere [7]. Cell growth potential was assayed by the MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)] test.

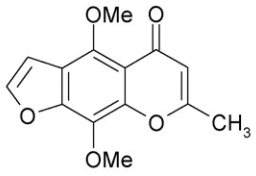
2.4. Morphological changes

To determine changes in cell size distribution, K562 cells were centrifuged onto a slide and then stained with May Grunwald and Giemsa. The cells were analyzed using a light microscope. Forward scatter and side scatter were measured by flow cytometric analysis to detect changes in size and granularity.

2.5. Cell cycle analysis

For flow cytometric analysis of DNA content, 5×10^5 exponentially growing K562 cells were irradiated with different





Khellin

Compounds	R1	R2	R3	R4	R5	R6	X	Y
Psoralen (Pso)	-H	-H	-H	-H	-H	-H	O	O
4-Methylpsoralen (4Me-Pso)	-H	$-CH_3$	-H	-H	-H	-H	O	O
5-Methylpsoralen (5Me-Pso)	-H	-H	$-CH_3$	-H	-H	-H	O	O
4,5'-Dimethylpsoralen (4,5'DiMe-Pso)	-H	$-CH_3$	-H	$-CH_3$	-H	-H	O	O
5,4'- Dimethylpsoralen (5,4'DiMe-Pso)	-H	-H	$-CH_3$	$-CH_3$	-H	-H	O	O
3,4- Dimethylpsoralen (3,4DiMe-Pso)	$-CH_3$	$-CH_3$	-H	-H	-H	-H	O	O
4,5',8-Trimethylpsoralen (TMP)	-H	$-CH_3$	-H	-H	$-CH_3$	$-CH_3$	O	O
5-Methoxypsoralen (5-MOP)	-H	-H	$-OCH_3$	-H	-H	-H	O	O
8-Methoxypsoralen (8-MOP)	-H	-H	-H	-H	-H	$-OCH_3$	O	O
5,8-Dimethoxypsoralen (5,8Di-MOP)	-H	-H	$-OCH_3$	-H	-H	$-OCH_3$	O	O
4,4'5'-Trimethyltiopsoralen (TMTP)	-H	$-CH_3$	-H	$-CH_3$	$-CH_3$	-H	O	S

Scheme 1 – Molecular structures of the linear psoralen derivatives.

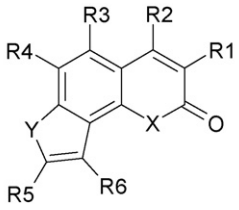
concentrations of the test compounds. After 24 and 48 h, the cells were centrifuged, fixed in ice-cold ethanol (70%), then treated with lysis buffer containing RNAseA, and finally stained with propidium iodide. Samples were analyzed on a Becton Coulter Epics XL-MCL flow cytometer. For cell cycle analysis, DNA histograms were analyzed using MultiCycle® for Windows (Phoenix Flow Systems, San Diego, CA).

2.6. *Externalization of phosphatidylserine*

Surface exposure of phosphatidylserine (PS) by apoptotic cells was measured by flow cytometry with a Coulter Cytomics FC500 (Becton Dickinson) by adding Annexin V-FITC to cells according to the manufacturer's instructions (Roche Diagnostic, Monza, Italy). Simultaneously, cells were stained with PI. Excitation was set at 488 nm and the emission filters were at 525 nm.

2.7. *Assessment of mitochondrial changes and reactive oxygen species production*

The mitochondrial membrane potential was measured with the lipophilic cation 5,5′,6,6′-tetrachlo-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine (JC-1, Molecular Probes Eugene, OR, USA) as described [17]. Briefly, 24 h after UVA irradiation, cells were collected by centrifugation and resuspended in HBSS containing 1 μM JC-1. The cells were then incubated for 10 min at 37 °C, centrifuged and resuspended again in HBSS. The production of reactive oxygen species (ROS) and the oxidation of cardiolipin were measured by flow cytometry using hydroethidine (HE, Molecular Probes Eugene, OR, USA), and 10-*N*-nonyl-acridine orange (NAO, Molecular Probes Eugene, OR, USA), respectively [17]. Briefly, 24 h after UVA irradiation, cells were collected by centrifugation and resuspended in HBSS containing the fluorescence probes HE, DCFDA or NAO at



Compounds	R1	R2	R3	R4	R5	R6	X	Y
Angelicin (Ang)	-H	-H	-H	-H	-H	-H	O	O
4-Methyl angelicin (4Me-Ang)	-H	$-CH_3$	-H	-H	-H	-H	O	O
4,5'-Dimethylangelicin 4,5'DiMe-Ang)	-H	$-CH_3$	-H	-H	$-CH_3$	-H	O	O
6,5- Dimethylangelicin (6,5DiMe-Ang)	-H	-H	$-CH_3$	$-CH_3$	-H	-H	O	O
4',5'- Dimethylangelicin (4',5'-DiMe-Ang)	-H	-H	-H	-H	$-CH_3$	$-CH_3$	O	O
4,5',4'-Trimethyangelicin (TMA)	-H	$-CH_3$	-H	-H	$-CH_3$	$-CH_3$	O	O
4,6,2'-Trimethylfuroquinolinone (TMFQ)	-H	$-CH_3$	-H	$-CH_3$	$-CH_3$	-H	NCH_3	O
4,2',3'-Trimethyltioangelicin (TMTA)	-H	$-CH_3$	-H	-H	$-CH_3$	$-CH_3$	O	S

Scheme 2 – Molecular structures of the angular psoralen derivatives.

the concentration of 2.5 μM, 5.0 μM and 100 nM, respectively. The cells were then incubated for 30 min at 37 °C, centrifuged and resuspended again in HBSS. The fluorescence was directly recorded with the flow cytometer using as excitation wavelength 488 nm and emission at 585 nm for HE and NAO.

2.8. *Caspases assay*

K562 cells were irradiated in the presence of the test compounds as described above, and after 24 h the cells were harvested, washed and resuspended in HBSS buffer containing the cell-permeable substrates for caspase-3 (FITC-DEVD-fmk, Bender Medsystem, USA), caspase-8 (FITC-IETD-fmk, Bender Medsystem, USA) and caspase-9 (FITC-LEDH-fmk, Bender Medsystem, USA), respectively. After 1 h of incubation at 37 °C, the cells were washed and analyzed by flow cytometry using the FL-1 channel.

2.9. *Human erythroid cell cultures from normal donors*

The two-phase liquid culture procedure was employed as previously described [8]. Mononuclear cells were isolated from peripheral blood samples of normal donors by Ficoll-Hypaque density gradient centrifugation and seeded in α-minimal essential medium supplemented with 10% FBS (Celbio, Milano, Italy), 1 μg/ml cyclosporine A (Sandoz, Basel, Switzerland), and 10% conditioned medium from the 5637 bladder carcinoma cell line [8]. The cultures were incubated at 37 °C, under an atmosphere of 5% CO_2 in air, with extra humidity. After 7 days incubation in this phase I culture, the nonadherent cells were harvested, washed, and then cultured in fresh medium composed of α-medium, 30% FBS, 1% deionized bovine serum albumin (BSA), 10^{-5} M β-mercaptoethanol, 1.5 mM L-glutamine, 10^{-6} M dexamethasone, and 1 U/ml human recombinant erythropoietin (EPO) (Inalco, Milano, Italy). This part of the culture is referred to as phase II. Compounds were added on day 6 of phase II. Cell samples were analyzed on day 9 of phase II [8].

2.10. *RNA isolation*

Total RNA was phenol–chloroform-extracted from cytoplasm of treated and untreated K562 cells as described elsewhere [8]. All solutions were made in diethylpyrocarbonate (DEPC)-treated water. The extracted RNA was precipitated in two volumes of absolute ethanol and stored at −80 °C, washed once with cold 75% ethanol, dried and dissolved in DEPC-treated water before use.

2.11. *Real-time quantitative RT-PCR*

Quantitative real-time PCR assay of γ-globin mRNA and α-globin transcripts have been carried out using gene-specific double fluorescently labelled probes in a 7700 Sequence Detection System version 1.6.3 (Applied Biosystems, Warrington Cheshire, UK). The following primer and probe sequences were used for real-time PCR: γ-globin forward primer, 5′-TGG CAA GAA GGT GCT GAC TTC-3′; γ-globin reverse primer, 5′-TCA CTC AGC TGG GCA AAG G-3′; γ-globin probe, 5′-FAM-TGG GAG ATG CCA TAA AGC ACC TGG-TAMRA-3′; α-globin forward primer, 5′-TCC CCA CCA CCA AGA CCT AC-3′; α-globin reverse primer, 5′-CCT TAA CCT GGG CAG AGC C-3′; α-globin probe, 5′-FAM-TCC CGC ACT TCG ACC TGA GCC A-TAMRA-3′. The reference sequences were ribosomal RNA and the endogenous control human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), where the probes were fluorescently labelled with VIC™ (Applied Biosystems) [3].

2.12. *Statistical analysis*

The statistical significance of difference in between different treatments was analyzed using one-way analysis of variance (ANOVA) and the Student–Newman–Keuls test. p values lower than 0.05 were considered statistically significant.

3. Results

3.1. *Psoralen derivatives reduce cell growth and induce erythroid differentiation*

We have investigated the effects of the combined treatment of K562 cells with a large series of structurally related linear and angular psoralen derivatives (Schemes 1 and 2) and UVA irradiation. The K562 cell line, which was established from a patient with chronic myelogenous leukemia in blast crisis [18], is a widely used model for the *in vitro* study of erythropoiesis and can be triggered to undergo differentiation by a variety of chemical agents such as hydroxyurea, butyric acid, mithramycin and hemin [2]. In the presence of these compounds, K562 cells develop phenotypical characteristics similar to those of normal red blood cells, including red blood cell surface antigens and hemoglobin synthesis. Since this cell line does not express p53 [19], it appears to be suitable for studies aimed at determining possible relationships between erythroid differentiation and induction of apoptosis independent from cellular changes of p53 expression.

The experimental procedure plan is based on the irradiation of the cells in the presence of the test compounds at different concentrations and different UVA doses. The analysis of cell growth efficiency and erythroid

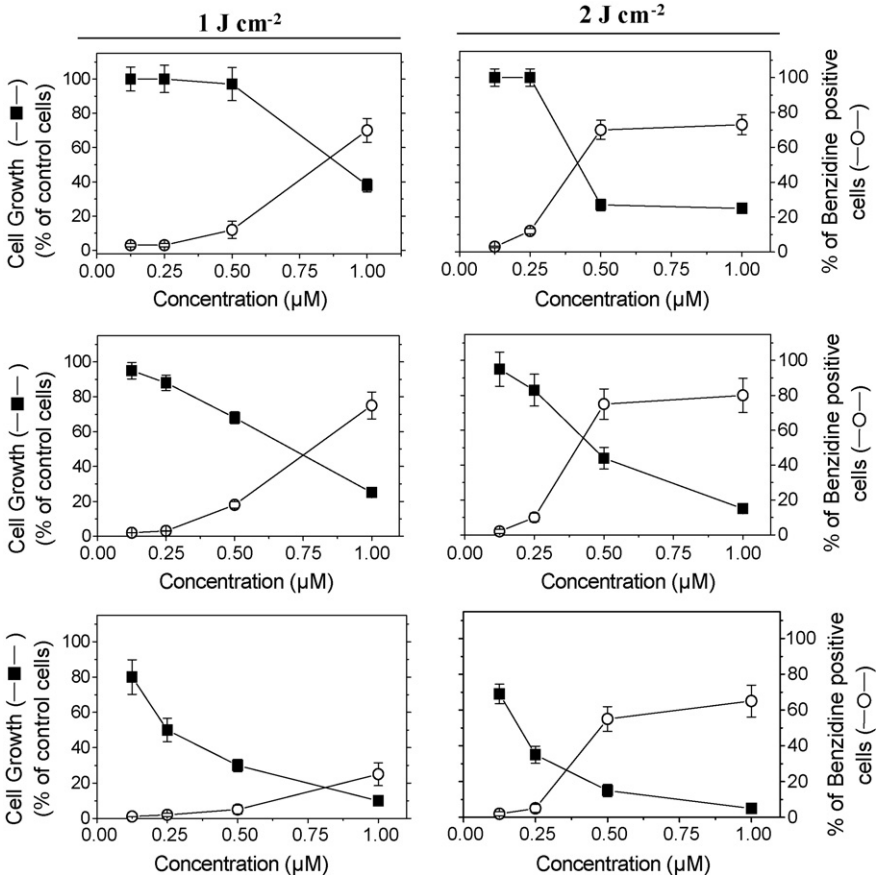


Fig. 1 – Time course of 5-MOP-induced cell growth inhibition and erythroid differentiation of K562 cells. Cell were irradiated with two UVA doses (1 and 2 J cm^{-2}) as indicated, in the presence of 5-MOP at different concentrations and then incubated for up to 7 days. Aliquots were removed at fifth day (upper panels), sixth day (middle panels) and seventh day (lower panels) for the determination of the number of viable and benzidine-positive cells.

Table 1 – Effects of linear psoralens on proliferation and differentiation of K562 cells

Treatment	Differentiation (% benzidine-positive cells)[a]			Cell growth (% of control)[a]		
	0 J cm^{-2}	1 J cm^{-2}	2 J cm^{-2}	0 J cm^{-2}	1 J cm^{-2}	2 J cm^{-2}
None	2	2	3	100	98	98
Psoralen (Pso), 1 μM	3	50	80	98	21	8
4Me-Pso, 1 μM	4	90	75	96	23	11
5Me-Pso, 1 μM	5	92	90	97	37	4
3,4-DiMe-Pso, 0.5 μM	3	25	28	95	25	3
4,5′-DiMe-Pso, 0.5 μM	5	61	67	96	27	8
5,4′-DiMe-Pso, 0.5 μM	4	15	2	96	9	2
4,8,5′-TMP, 0.25 μM	3	68	95	97	10	3
5-MOP, 1 μM	3	75	80	95	38	27
8-MOP, 1 μM	2	5	15	98	98	75
5,8-DiMOP, 1 μM	4	10	12	96	98	66
TMTP, 1 μM	3	20	50	95	58	11
Khellin, 1 μM	2	20	20	98	98	73

[a] The data reported represent the effects on erythroid differentiation (percentage of benzidine-positive cells) and cell growth (percentage of control) after 6 days from the UVA irradiation at the indicated concentrations and UVA doses, from at least three independent experiments. Experimental errors never exceed 20%.

differentiation was carried out at the 5–7 days after the treatment.

Fig. 1 shows a representative experiment carried out with 5-methoxypsoralen (5-MOP) at two UVA doses, 1 and 2 J cm^{-2}, respectively. A concentration-dependent reduction of cell growth can be observed, while in the meantime a marked differentiation was also observed. In particular, this effect was maximum at the sixth day (Fig. 1, middle panels).

In Tables 1 and 2 are reported the data obtained with linear and angular derivatives analyzed at the sixth day obtained at the highest concentrations. It can be observed that the lead compound psoralen, the two mono methylated derivatives, 4-methyl psoralen and 5-methyl psoralen, the dimethylated derivative 4,5-dimethylpsoralen and 5-MOP are the most interesting compounds, demonstrating high percentages of benzidine-positive cells when used at low concentrations and low UVA dose. At the same time, all the compounds caused a reduction in cell growth, in particular at the UVA dose of 2 J cm^{-2}. At this UVA dosage, however, the lowest anti-proliferative effect was reproducibly displayed by 5-MOP (Table 1). It is important to underline that in the absence of UVA irradiation, cells treated with all the compounds exhibited a cell growth ability and a proportion of Hb-containing (benzidine-positive) cells similar to that of the irradiated control cells.

Interestingly, the 5,8-dimethoxypsoralen and the furochromone derivative khellin, which do not intercalate into DNA exhibiting after UVA irradiation a poor DNA photobinding [20], do not produce significant effects in terms of differentiation and reduction of cell growth ability, suggesting that the DNA photodamage induced by psoralens could be involved in the observed induction of the differentiation process. Thus, the intercalation and the consequent photobinding to the DNA seem to play a major role both in terms of reduction of cell growth and induction of differentiation although other types of cell damage could be important.

Among the angular derivatives (Table 2), the most interesting compounds appear to be angelicin and the trimethyl derivative TMA, although their activity is reduced in comparison to the linear ones.

Table 2 – Effects of angular psoralens on proliferation and differentiation of K562 cells

Treatment	Differentiation (% benzidine-positive cells)[a]			Cell growth (% of control)[a]		
	0 J cm^{-2}	1 J cm^{-2}	2 J cm^{-2}	0 J cm^{-2}	1 J cm^{-2}	2 J cm^{-2}
None	3	2	3	100	98	98
Angelicin (Ang), 1 μM	4	41	78	97	20	15
4Me-Ang, 1 μM	3	16	18	96	68	27
4,5-DiMe-Ang, 1 μM	3	20	25	97	78	62
6,5-DiMe-Ang, 1 μM	2	20	28	96	50	30
4,5′-DiMe-Ang, 1 μM	4	21	38	98	66	48
TMA, 0.5 μM	4	30	37	95	23	16
TMTA, 0.1 μM	3	4	4	95	80	41
TMFQ, 0.025 μM	2	2	3	96	11	3

[a] The data reported represent the effects on erythroid differentiation (percentage of benzidine-positive cells) and cell growth (percentage of control) after 6 days from the UVA irradiation at the indicated concentrations and UVA doses, from at least three independent experiments. Experimental errors never exceed 20%.

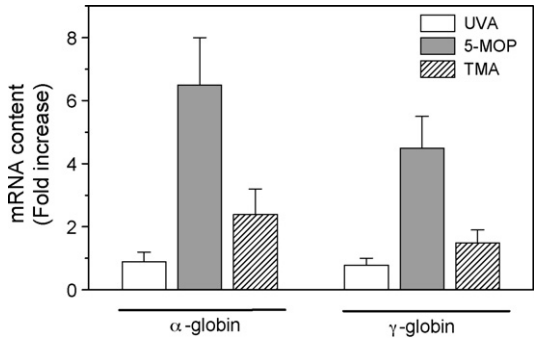


Fig. 2 – Increase in α-globin and γ-globin mRNA content in K562 cells irradiated (2 J cm^{-2}) in the presence of 5-MOP and TMA at the concentrations of 1 and 0.5 μM, respectively. Expression of globin genes was assayed by reverse transcriptase polymerase-chain reaction (RT-PCR) after 6 days from the irradiation.

From these results, we selected two derivatives, one linear (5-MOP) and one angular (TMA), which present good erythroid inducing ability associated with lower anti-proliferative effects.

3.2. *Irradiation of K562 cells with 5-MOP and TMA induces increase of α-globin and γ-globin mRNA content*

The effect of 5-MOP and TMA on the expression of globin genes in K562 cells was investigated by quantitative RT-PCR. Cells were either untreated, treated with UVA or with UVA (2 J cm^{-2}) plus either 5-MOP or TMA at the concentrations of 1 and 0.5 μM, respectively. The results (Fig. 2) clearly indicate that, as expected from the data indicating a sharp increase of benzidine-positive cells (Tables 1 and 2), accumulation of both α-globin and γ-globin mRNA is increased following treatment. Accumulation of β-globin mRNA was not analyzed, since K562 cells do not express this gene [2]. The changes of globin gene expression (RT-PCR analysis) and the increase of the proportion of hemoglobin-containing cells (benzidine test), clearly confirm that the data concerning cell growth (obtained with the MTT test) indicate, apart from overall cytotoxicity, modifications in cell growth associated with the activation of erythroid differentiation.

Accordingly, cell morphology, cell cycle parameters and apoptosis were assessed in K562 cells after 5–7 days of treatment.

3.3. *Irradiation of K562 cells with 5-MOP and TMA affects cell morphology*

The effect of 5-MOP and TMA on K562 cells was further investigated by morphological analysis using light microscopy and flow cytometry. As it can be observed from Fig. 3 (panel A), several larger cells were clearly visible after irradiation with 5-MOP. These results were in good agreement with those obtained by FACS analysis. Changes in granularity measured by side scatter (SSC on X-axis) were observed in K562 cells treated with 5-MOP and TMA for 24 and 48 h after the irradiation and again the most pronounced cell size change measured by forward scatter (FS on Y-axis) was visible (Fig. 3, panel B). This finding is not unexpected, since alteration of K562 cell size has been reported using several inducers, such as cytosine-arabinoside, doxorubicine and imatinib [21,22].

3.4. *Induction of differentiation of K562 cells is accompanied by arrest in the G2/M phase of the cell cycle*

To further characterize the effects of 5-MOP and TMA on K562 cell cycle, flow cytometry analysis was performed 24 and 48 h after irradiation on propidium iodide stained cells. Flow cytometric analysis of DNA content indicates that UVA irradiation of K562 cells treated with increasing concentrations of 5-MOP and TMA led to profound changes of the cell cycle profile (Fig. 4). In particular, as it can be observed in Table 3, untreated cells showed a classical pattern of proliferating cells proportionally distributed in the G1, S and G2/M phases of the cell cycle.

On the contrary, a very clear G2/M arrest pattern was observed, for both compounds, at the concentrations of 0.125 and 0.25 μM (Fig. 4), with a concomitant decrease of all the other phases of the cell cycle. At later times after irradiation (48 h) a hypo-diploid DNA content peak (sub-G1), reflecting fragmented DNA can be observed, indicating apoptosis of the cells. At high concentrations (0.5 and 1.0 μM) the pattern is disturbed, due to the presence of a relevant proportion of apoptotic cells distributed along the different phases of the

Table 3 – Percentage of cell cycle phases of K562 cells after irradiation with 5-MOP (0.25 μM) and TMA (0.125 μM) irradiation of 5-MOP and TMA on the cell cycle of K562 cells

	% G1	G2/M	S[a]	Apoptotic cells[b] (sub-G1)
Dark (24 h)	28.4	3.9	66.3	0.4
UVA alone (24 h)				
1 J cm^{-2}	31.8	2.3	65.8	0.5
2 J cm^{-2}	27.9	8.5	63.5	0.1
5-MOP (24 h)				
1 J cm^{-2}	7.0	66.0	27.0	0.3
2 J cm^{-2}	1.8	79.8	18.4	1.4
TMA (24 h)				
1 J cm^{-2}	0.6	29.6	69.8	0.8
2 J cm^{-2}	0.2	46.5	53.3	0.3
Dark 48 h	36.2	5.2	58.1	1.7
UVA alone (48 h)				
1 J cm^{-2}	40.1	11.8	48.2	0.4
2 J cm^{-2}	41.5	12.2	46.3	0.4
5-MOP (48 h)				
1 J cm^{-2}	29.7	28.0	42.3	7.1
2 J cm^{-2}	5.5	69.3	25.3	14.3
TMA (48 h)				
1 J cm^{-2}	14.0	39.7	46.3	10.1
2 J cm^{-2}	8.7	54.1	37.2	18.2

[a] The percentage of each phase of the cell cycle (G1, S and G2/M) was calculated on living cells.
[b] The percentage of apoptotic cells is referred to as cell populations characterized by the appearance of sub-G1 peak.

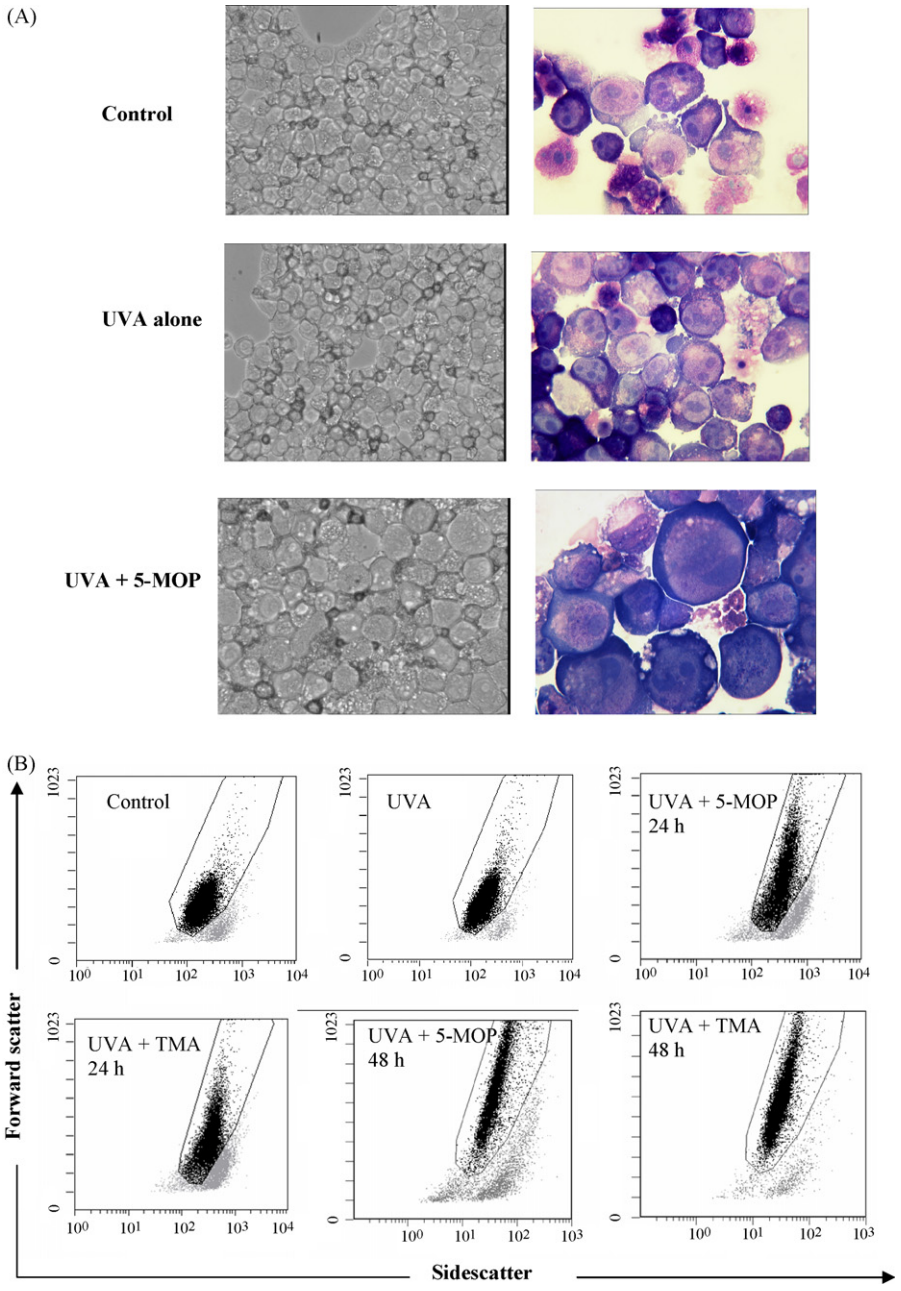


Fig. 3 – Morphological changes of K562 after irradiation in the presence of 5-MOP and TMA, respectively. Panel A. Light microscopy analysis: K562 cells were irradiated (2 J cm^{-2}), with 5-MOP and TMA at the concentrations of 1 and 0.5 μM, respectively and after 48 h cells were photographed directly (left panels) or after staining with May-Grunwald Giemsa stain (right panels). Panel B. Flow cytometry was used to show change in size (forward scatter on the Y-axis) and granularity (side scatter on the X-axis) of K562 cells after 24 and 48 h after the irradiation in the presence of 5-MOP (1 μM) and TMA (0.5 μM).

cell cycle (Fig. 4). This was reproducibly observed in three independent experiments. Therefore, for quantitatively determining the effects of 5-MOP and TMA on the different phases of the cell cycle, only the results obtained with 0.125 and 0.25 μM concentrations were analyzed and reported in Table 3.

After UVA irradiation of 5-MOP and TMA treated K562, cells by alterations of cell cycle were accompanied by the appearance of sub-G1 cells, indicating possible activation of apoptosis. Interestingly, several inducers of erythroid differentiation are also able to induce apoptosis, such as cisplatin [23], tallimustine [7], imatinib [22], or the topoisomerase II inhibitor Dexrazoxane [24].

3.5. *Loss of plasma membrane asymmetry during apoptosis*

To characterize psoralen-induced apoptosis, we performed a biparametric cytofluorimetric analysis using propidium iodide (PI) and Annexin-V-FITC, which stain DNA and phosphatidylserine (PS) residues, respectively [25]. Annexin-V is a

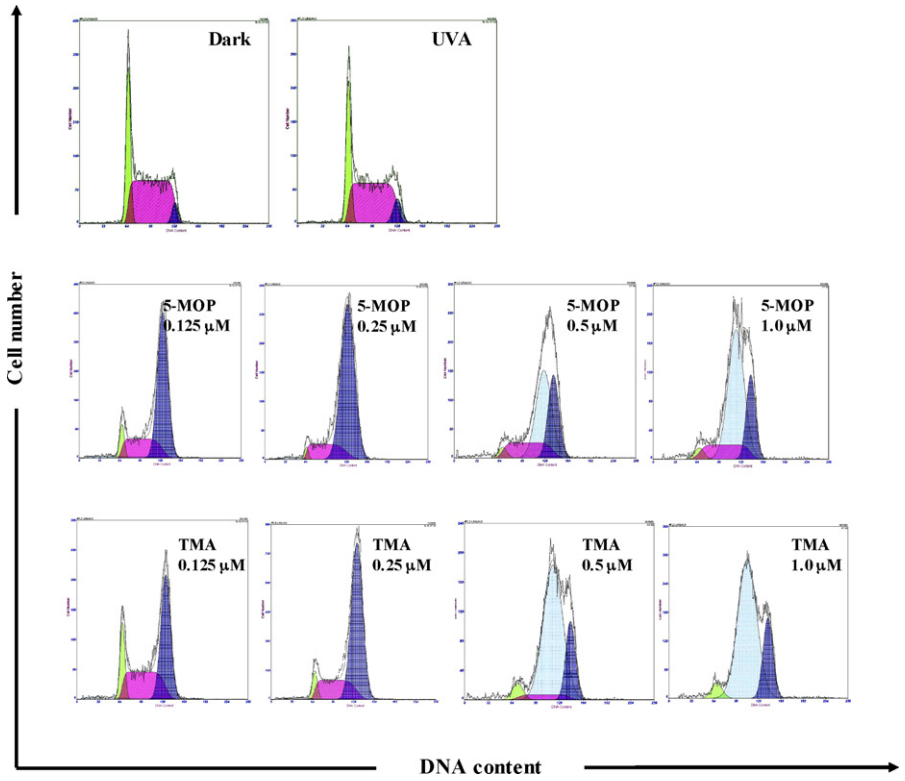


Fig. 4 – Representatives histograms of flow cytometry data of K562 cells after irradiation in the presence of 5-MOP and TMA. Cells were irradiated (2 J cm^{-2}), at the indicated concentrations of 5-MOP and TMA and after 24 h of incubation were labelled with propidium iodide and analyzed by flow cytometry as described in Section 2.

Ca^{2+}-dependent phospholipid-binding protein with high affinity for PS. Annexin-V staining precedes the loss of membrane integrity which accompanies the latest stages of cell death resulting from either apoptotic or necrotic processes [26]. Because externalization of PS occurs in the earlier stages of apoptosis, Annexin-V staining identifies apoptosis at an earlier stage than sub-G_1 appearance, which represents a later stage of apoptosis, involving nuclear changes such as DNA fragmentation.

After 24 h from the irradiation with 5-MOP and TMA, K562 cells were labelled with the two dyes, washed and the resulting red (PI) and green (FITC) fluorescence was monitored by flow cytometry. Both compounds induced a significant increase of the proportion of apoptotic cells after 24 and 48 h after irradiation (Fig. 5). The percentage of Annexin-V positive cells increased gradually upon treatment in a time- and dose-dependent manner. It is interesting to note that also at the highest concentration used, the percentage of apoptotic cells reach the values of 25–35% for both compounds. These findings prompted us to further investigate the apoptotic machinery in K562 cells after treatment with the two compounds.

3.6. *Variations of the mitochondrial potential induced by psoralen derivatives*

Mitochondria play an essential role in the propagation of apoptosis [27,28]. It is well established that, at an early stage, apoptotic stimuli alter the mitochondrial transmembrane potential ($\Delta\psi_{mt}$). To address whether the tested compounds affected the $\Delta\psi_{mt}$, we monitored the fluorescence of the dye JC-1, which is considered a reliable probe to assess $\Delta\psi_{mt}$ [29]. JC-1 has the remarkable property of forming red fluorescent aggregates locally and spontaneously under high mitochondrial $\Delta\psi_{mt}$, whereas the monomeric form fluoresces in green. Irradiated K562 cells in the presence of 5-MOP and TMA exhibited a shift in fluorescence in a dose-dependent manner, compared to the control cells, indicating depolarisation of mitochondrial membrane potential. The percentage of cells with low mitochondrial potential for the test compounds is depicted in Figs. 6 and 7 (upper panels). It is interesting to note that the disruption of $\Delta\psi_{mt}$ is associated with the appearance of Annexin-V positivity in the treated cells. In fact, the dissipation of $\Delta\psi_{mt}$ is characteristic of apoptosis and has commonly been observed with different drugs, irrespectively of the cell type [27].

3.7. *Mitochondrial generation of ROS and oxidation of cardiolipin*

Mitochondrial membrane depolarisation is associated with mitochondrial production of reactive oxygen species (ROS) [30]. We have, therefore, investigated whether ROS production increases after treatment with the test compounds. We employed the fluorescence indicator hydroethidine (HE), whose fluorescence appears if reactive oxygen species are

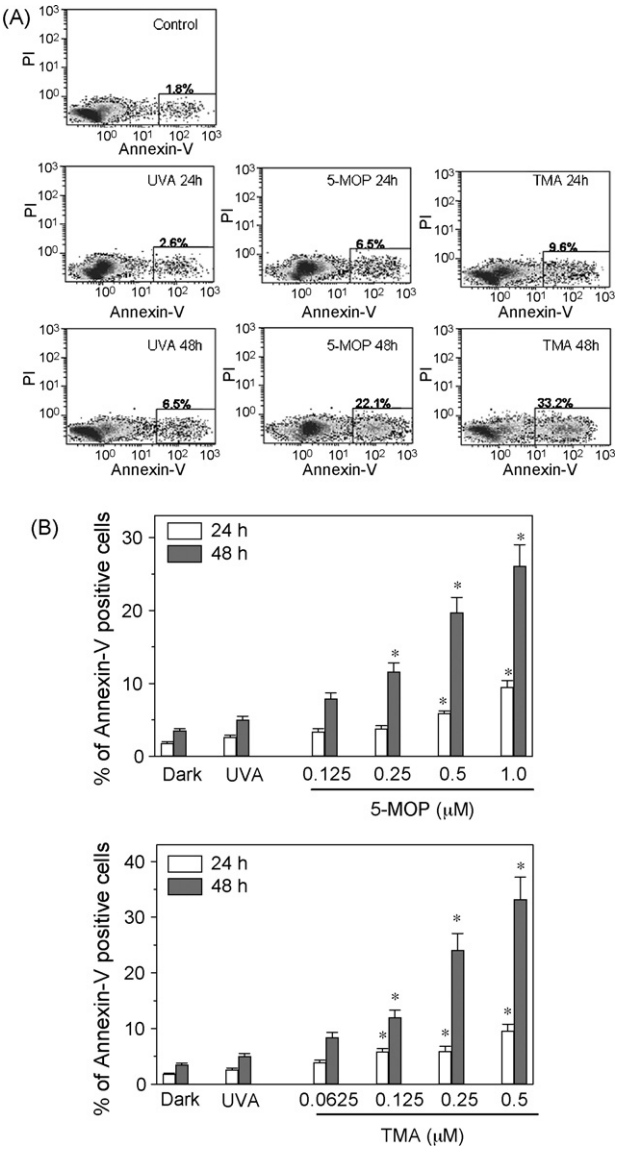


Fig. 5 – Panel A. Representative biparametric histograms of K562 cells after irradiation in the presence of 5-MOP and TMA. Cells were irradiated (2 J cm^{-2}), with 5-MOP (1 μM) and TMA (0.5 μM), and then stained with PI and Annexin-V-FITC after 24 and 48 h from the treatment. Panel B. Percentage of Annexin-V positive cells for the 5-MOP and TMA after 24 and 48 h from the irradiation at different concentrations. Data are expressed as mean ± S.E.M. of three independent experiments. *$p < 0.05$ (Newman–Keuls t-test).

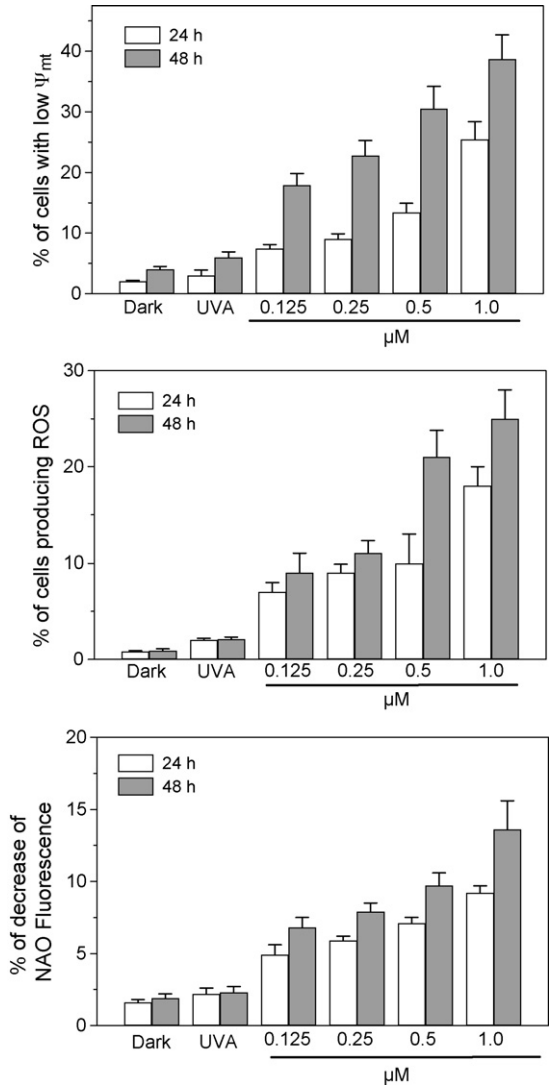


Fig. 6 – Assessment of mitochondrial dysfunction after irradiation (2 J cm^{-2}), of K562 cells with 5-MOP at different concentrations. Upper panel: induction of loss of mitochondrial membrane potential after 24 and 48 h from the irradiation. Cells were stained with the fluorescent probe JC-1. Middle panel: mitochondrial production of ROS in K562 cells. After 24 and 48 h from the irradiation with 5-MOP, cells were stained with hydroethidine and analyzed by flow cytometry. Lower panel: decrease of mean fluorescence intensity of the cardiolipin-binding dye NAO in K562 cells after 24 and 48 h from the irradiation with 5-MOP. Data are expressed as mean ± S.E.M. of three independent experiments.

generated. HE is oxidized by superoxide anion into ethidium ion, which emits red fluorescence [31]. Superoxide is produced by mitochondria due to a shift from the normal four-electron reduction of O_2 to a one-electron reduction when cytochrome c is released from mitochondria.

The results are presented in Figs. 6 and 7 (middle panels), where it can be observed that both 5-MOP and TMA remarkably induce the production of ROS in comparison to control cells, in particular after 48 h after treatment.

In parallel, we evaluated the damage produced by ROS in mitochondria by assessing the oxidation state of cardiolipin, a phospholipid restricted to the inner mitochondrial membrane, using the specific dye 10-*N*-nonyl-acridine orange (NAO) [32]. This fluorescent probe specifically binds to cardiolipin, and its binding affinity and fluorescence properties depend on the oxidation state of cardiolipin. NAO binds with high affinity to a non-oxidized cardiolipin in a 2:1 ratio, whereas in the case of oxidized cardiolipin, NAO has been

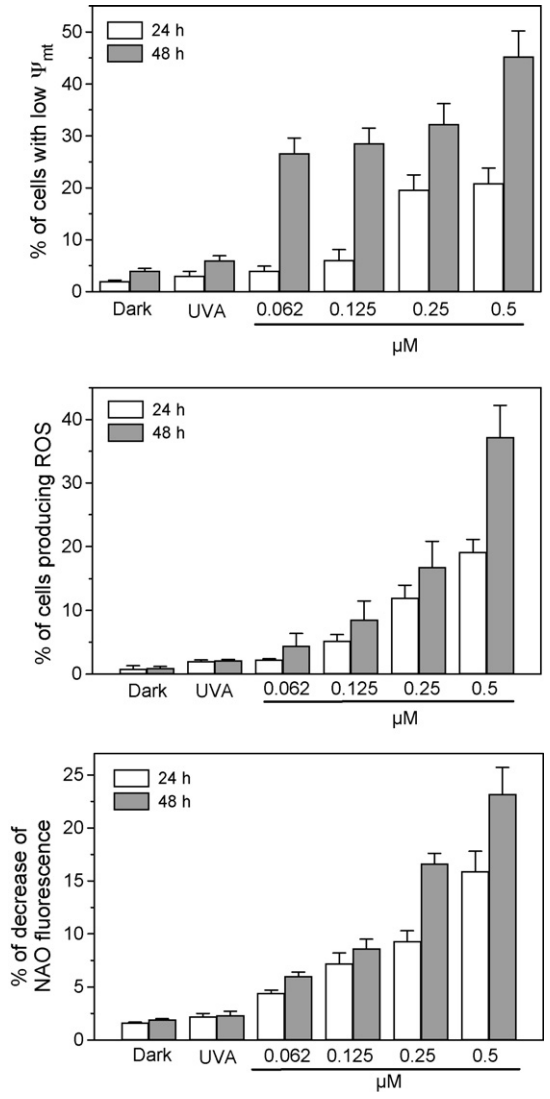


Fig. 7 – Assessment of mitochondrial dysfunction after irradiation (2 J cm^{-2}), of K562 cells with TMA at different concentrations. Upper panel: induction of loss of mitochondrial membrane potential after 24 and 48 h from the irradiation. Cells were stained with the fluorescent probe JC-1. Middle panel: mitochondrial production of ROS in K562 cells. After 24 and 48 h from the irradiation with TMA, cells were stained with hydroethidine and analyzed by flow cytometry. Lower panel: decrease of mean fluorescence intensity of the cardiolipin-binding dye NAO in K562 cells after 24 and 48 h from the irradiation with TMA. Data are expressed as mean ± S.E.M. of three independent experiments.

reported to bind less to this phospholipid with a decreased activity, reflected by lower fluorescence intensity [33]. Therefore, oxidative stress localized to mitochondria can be assessed by measuring fluorescence of NAO. K562 cells irradiated with 5-MOP and TMA showed (Figs. 6 and 7, lower panels) a progressive and remarkable decrease in mean NAO fluorescence, with increasing concentration of the two compounds, consistent with a loss in cardiolipin content. Altogether, these results strongly suggest that both 5-MOP and TMA induce mitochondria-dependent apoptosis of K562 cells.

3.8. *Caspase activation by psoralens*

Caspases are the central executioners of apoptosis mediated by various inducers, and they are synthesized as proenzymes activated by cleavage [34]. Caspase-2, -8, -9, and -10 (apical caspases) are usually the first to be stimulated in the apoptotic process and then activate effector caspases such as caspase-3 [35].

To investigate the role of caspases activation in programmed cell death mediated by 5-MOP and TMA, we analyzed K562 cells 24 h after irradiation by flow cytometry. The cells were assayed for an activity capable of cleaving DEVD-FITC, LEDH-FITC and IETD-FITC, respectively, using a flow cytometry assay. DEVD-ase activity is indicative of caspase-3 cleavage, whereas LEDH-ase activity is indicative of caspase-9 cleavage activity, and IETD-ase is indicative of caspase-8 activity.

Irradiation of K562 cells in the presence of 5-MOP and TMA was found to activate both initiator and executor caspases, as shown in Fig. 8. In particular, caspase-3 and caspase-9 are the most activated, suggesting that the mitochondrial apoptotic pathway plays a major role during psoralen mediated induction of apoptosis of K562 cells.

3.9. *DNA and mitochondrial damage are involved in psoralen-induced erythroid differentiation of K562 cells*

With the purpose to investigate in a preliminary way the mechanism of action of psoralen derivatives on the induction of erythroid differentiation, we have performed some experiments in the presence of different pharmacological inhibitors.

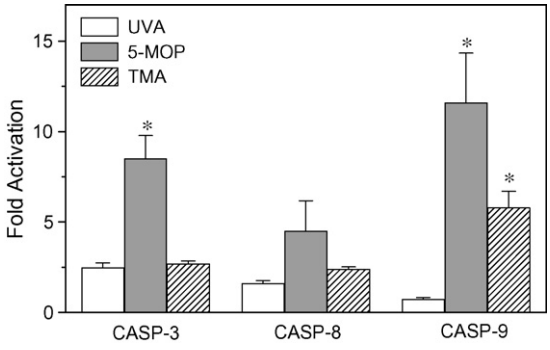


Fig. 8 – Caspase-3, -8 and -9 induced activity in K562 cells after irradiation in the presence of 5-MOP and TMA. K562 cells were irradiated (2 J cm^{-2}) in the presence of 5-MOP (1 μM) and TMA (0.5 μM). After 24 h, cells were harvested and assayed by flow cytometry for caspase-3 activity using the caspase-3 substrate DEVD-FITC, caspase-8 using the substrate IETD-FITC and caspase-9 using the substrate LEHD-FITC. Data are represented as fold increase of activity of the enzymes normalized to total protein, in comparison to the control. Data are expressed as mean ± S.E.M. of three independent experiments. *$p < 0.05$ (Newman–Keuls t-test).

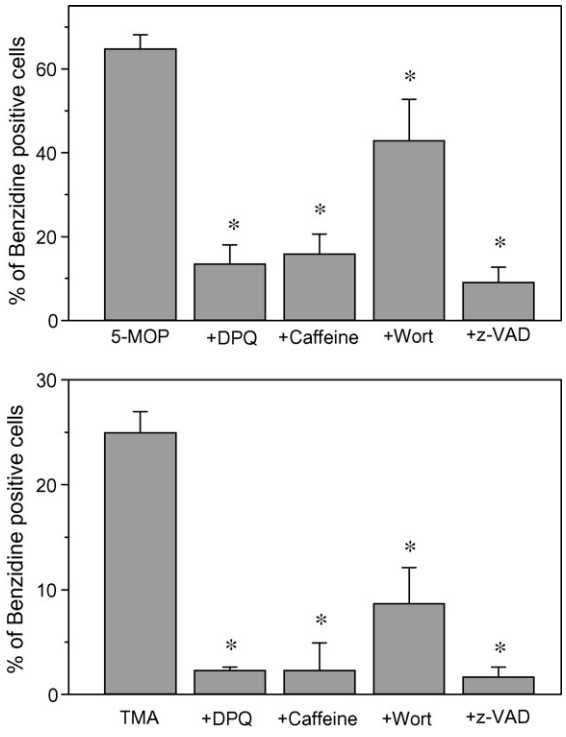


Fig. 9 – Effect of caffeine (2 mM), wortmannin (10 μM), DPQ (30 μM) and z-VAD.fmk (50 μM) on psoralen-induced erythrodifferentiation on K562 cells. Cell were irradiated (2 J cm^{-2}) with 5-MOP (upper panel) and TMA (lower panel) at the concentration of 1 and 0.5 μM, respectively, and at the sixth day cell was harvested and stained with benzidine. The different compounds were added simultaneously to 5-MOP and TMA. Data are expressed as mean ± S.E.M. for five experiments *$p < 0.05$ (Newman–Keuls *t*-test).

The best-known intracellular target of psoralen is of course DNA. The irradiation of cells in the presence of psoralens leads to DNA damage and this results in the initiation of the down stream cellular response to DNA damage. In mammals, the ATM (ataxia-teleangiectasia-mutated) and ATR (ATM and Rad3 related) protein kinases function at the top of the signal transduction cascade that is triggered by DNA damage [36,37]. To assess whether psoralen-induced erythroid differentiation of K562 cells is mediated by ATM or ATR, we examined the production of hemoglobin by benzidine staining in the presence of caffeine, a well-known inhibitor of ATM and ATR, but not of DNA-dependent protein kinase [38,39].

As depicted in Fig. 9, caffeine significantly inhibits the production of hemoglobin in cells irradiated both in the presence of 5-MOP and TMA 6 days after UVA irradiation.

DNA damage is also recognized and results in the activation of DNA-dependent protein kinases. DNA-PK is a member of PI3-kinase family and thereby activates p53. Wortmannin is an inhibitor of the catalytic subunit of the PI3-kinase family of enzymes including DNA-PK [40]. As clearly evident from Fig. 9, also wortmannin reduces significantly the induction of differentiation mediated by 5-MOP and TMA.

Another intracellular target of psoralen involved in the cellular death is represented by mitochondria. To explore the involvement of the mitochondrial pathway via activation of caspase-3 and caspase-9, K562 cells were irradiated in the presence of the pancaspase inhibitor z-VAD.fmk and erythroid differentiation was evaluated by benzidine assay. As shown in Fig. 9, z-VAD.fmk suppress erythroid differentiation induced by both 5-MOP and TMA.

Poly-ADP-ribose polymerase-1 (PARP-1) is a nuclear enzyme that responds to DNA damage and facilitates DNA repair [41]. The specific PARP inhibitor, 3,4-dihydro-5-[4(1piperidinyil)butoxy]-1(2*H*)-isoquinolinone (DPQ) [42], completely blocks erythroid differentiation induced by psoralen.

3.10. *UVA-irradiated erythroid precursor cells from healthy subjects treated with 5-MOP and TMA: increase of production of γ-globin mRNA*

In order to propose the UVA irradiation of psoralen treated cells as a strategy for future experiments aimed at determining possible applications in the therapy of β-thalassemia, it is very important to determine the effects of this treatment on primary human erythroid cells. In fact, not all the inducers of K562 cells are active when tested on erythroid cells isolated from human donors or β-thalassemic patients [2]. To study the effects of UVA irradiation with 5-MOP and TMA on differentiation of human erythroid progenitor cells, we employed the two-phase liquid culture system as described elsewhere [8]. In this procedure, early erythroid committed progenitors (BFUe) derived from the peripheral blood proliferate and differentiate during phase I (in the absence of EPO) into late progenitors (CFUe). In phase II, in the presence of EPO, the latter cells continue their proliferation and mature into Hb-containing orthochromatic normoblasts [2]. 5-MOP and TMA were added on day 4 of phase II and irradiated with UVA light. The results of quantitative RT-PCR analysis carried out after 4

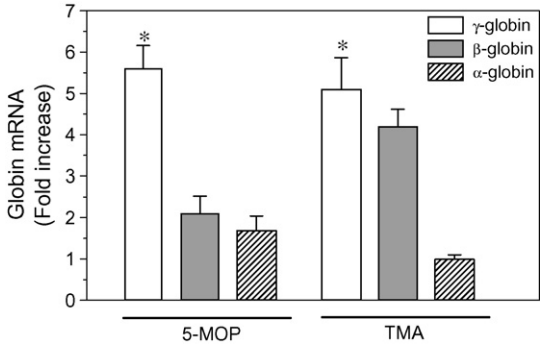


Fig. 10 – Effect on globins mRNA content of irradiation (2 J cm^{-2}) of human erythroid precursor cells treated with 5-MOP and TMA at the concentrations of 1 and 0.5 μM, respectively. Expression of globin genes was assayed by reverse transcriptase polymerase-chain reaction (RT-PCR) using RNA isolated 6 days after the irradiation. Data are expressed as mean ± S.E.M. of independent experiments performed on erythroid precursors isolated from the peripheral blood obtained from three donors (*$p < 0.05$ γ-globin vs. α-globin).

days from the irradiation are shown in Fig. 10 and suggest an increase of β-globin and γ-globin mRNA following UVA irradiation of 5-MOP and TMA treated cells. UVA irradiation of untreated control cells does not lead to changes of globin gene expression. In these experiments, GAPDH was used as reference RNA sequence. Interestingly, a slight preferential accumulation of γ-globin mRNA with respect to β-globin mRNA is detectable, whereas α-globin mRNA content does not change. The differences between expression of γ-globin and β-globin mRNAs and expression of α-globin mRNA are statistically significant ($p < 0.05$). In consideration of the excess of α-globin mRNA present in β-thalassemia patients, the finding that the reported treatments do not alter the expression of α-globin genes is of interest, as underlined in studies performed with other inducers [2,3,8].

4. Discussion

Pharmacologically mediated stimulation of human γ-globin gene expression and increase of fetal hemoglobin (HbF) levels is a potential therapeutic modality in haematological disorders, including β-thalassemia and sickle cell anemia [2]. Accordingly, several novel inducers were described able to increase HbF production and expression of γ-globin genes [2,8,43]. In this respect, pharmacological therapy of β-thalassemia is expected to be crucial for several developing countries, unable to efficiently sustain the high-cost clinical management of β-thalassemia patients requiring regular transfusion regimen, chelation therapy and advanced hospital facilities [2]. On the other hand, large investments by pharmaceutical companies finalized to the design, production and testing of novel drugs for the treatment of β-thalassemia are discouraged by the fact that this pathology is a rare disease in developed countries, due to the recurrent campaigns for prevention, genetic counseling and prenatal diagnosis [2]. Therefore, the search of molecules, already employed for unrelated pathologies, but exhibiting the property of inducing γ-globin gene expression, is of great interest.

In the field of HbF induction, furocoumarins appears to be appealing molecules. We have already published that angelicin is a powerful inducer of HbF production in human cultured erythroid precursor cells [16]. Therefore, psoralen-like molecules should be considered, as mithramycin [8], rapamycin [3] and everolimus [43] as a possible class of compounds to be used in the pharmacological experimental therapy of β-thalassemia.

The major finding of this manuscript is that exposure of erythroid cells to UVA, when combined with treatment with furocoumarins, leads to strong and reproducible effects on cell cycle parameters (G2 arrest), followed by induction of apoptosis, in association with the activation of the induction of the erythroid differentiation program and high level of accumulation of globin mRNA. These effects were demonstrated in two experimental cell systems, the human leukemia K562 cell line and the two-phase liquid culture of human erythroid progenitor cells isolated from healthy volunteers.

On these models we have evaluated a large series of linear and angular psoralen derivatives for their ability to induce erythroid differentiation. Linear derivatives appear to be the most powerful agents able to induce erythroid differentiation, as clearly demonstrated by both benzidine staining and increase of β- and γ-globin mRNA content. This effect could be related to the ability of these compounds to form DNA interstrand cross-links, since angular derivatives which are not able to form cross-links present a lower activity. However, the angular derivative angelicin, known to photoinduce only DNA monoadducts, is endowed with a remarkable activity, both in terms of induction of erythroid differentiation and reduction of cell growth. One possible factor contributing to this activity is cellular uptake and distribution inside the cell of this derivative. Despite the fact that further studies are necessary to fully explain the possible different mechanism of action of linear and angular derivatives, high biological activity of angelicin, in respect to linear psoralens has been already evidenced in previous studies carried out on different cell lines [17,44]. From a comparative point of view it is interesting to note that 5-MOP and other derivatives are able to induce erythroid differentiation at lower concentrations with respect to other known inducers such as hydroxyurea, butyric acid and Ara-C [2].

Irradiation of cells with 5-MOP or TMA induce a growth arrest in G2/M phase along with a concomitant decrease in G1 and S phase. This effect was also seen for Ara-C and doxorubicin induced K562 cell differentiation [21,45].

In an attempt to identify the potential mechanism(s) of action, we have demonstrated that both caffeine, an inhibitor of ATM/ATR, and wortmannin, an inhibitor of PI3-kinase, significantly inhibits the induction of differentiation, suggesting that DNA double strand breaks generated as repaired intermediates of psoralen photoproducts in DNA may be associated with the induction of differentiation. This evidence suggests a mechanism of action similar to that observed in Ara-C induced K562 cell differentiation [45]. Our results are compatible with a psoralen-UVA induced stalling of the replication forks (as suggested also for other HbF inducers, such as hydroxyurea), associated with DNA break formation leading to activation of kinases such as ATM/ATR and PI3-kinase.

Recent reports pointed out the relevance of the photodamage induced by psoralen at the mitochondrial level [44,46]. In particular, TMA induces higher mitochondrial damage than 5-MOP, in agreement with previous finding of our group [44,46]. Interestingly, both z-VAD.fmk (a pancaspase inhibitor) and DPQ (an inhibitor of PARP-1) also reduce the differentiation of K562 cells, suggesting that also the mitochondrial damage induced by irradiated psoralen could be involved in the mechanism of differentiation.

Thus, an interesting possibility is that psoralens exert a double action both on mitochondria and on the nucleus. While the effect of psoralens at the nuclear level is well known and characterized, little information is available on the action of psoralens on mitochondria in mammalian cells. As far as the effects on K562 cells are concerned, since these cells are unable to express p53, the effects described are clearly independent of p53.

In our opinion, the results presented in this manuscript are of interest from several points of view. Transcriptional activation of the expression of human β- and γ-globin genes is an important step in pharmacological treatment of β-

thalassemia, in association with specific β-thalassemia genotypes. As far as the induction of γ-globin mRNA is concerned, it is well known that activation of fetal globin genes might reproduce the HPFH phenotype, thereby ameliorating the clinical parameters of β-thalassemia patients. The induction of β-globin genes might be important for several β-thalassemia phenotypes, including those caused by altered splicing of pre-mRNA and mutations present within the β-globin promoter. In addition, these data introduce the interesting possibility of performing photopheresis-like treatment with psoralen derivatives including (a) isolation of white cells from the patient, (b) UVA irradiation of cells and (c) re-infusion of PUVA-treated cells. In fact, the UVA doses employed here were comparable to those already used in photopheresis [14,15].

Accordingly, our data strongly encourage further experiments employing erythroid precursor cells from β-thalassemia patients exhibiting different genotype/phenotype, as already performed by our research group for other HbF inducers [3,8]. In addition, several interesting *in vivo* experimental systems have been described in the last 20 years [47], reproducing the β-thalassemia [48,49] and the sickle cell anemia (SCA) [50,51] phenotypes. These model systems were employed to test molecules suitable for therapy. For instance, hydroxyurea, either alone [52] or used in combination therapy with erythropoietin and clotrimazole [53] was employed for ameliorating the erythroid parameters in a β-thalassemic mouse system. The use of SCA transgenic mice as a model system to detect anti-sickling molecules was reported by Iyamu et al. using hydroxyurea [54] and Niprisan [55]. Moreover, several humanized transgenic mice systems have been recently reported and might be extremely useful for *in vivo* studies [56,57]. In addition, the study of the effects on hematopoietic cells of different lineages might be proposed [58]. Finally, the effects on *in vivo* patients might be considered when employing hydroxyurea [59]. In this respect, it could be very interesting to determine whether photopheresis is beneficial *in vivo* and modifies the expression of the β-like globin genes, as reported in the present study.

Acknowledgements

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