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Induction of erythroid differentiation and increased globin mRNA production with furocoumarins and their photoproducts

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ABSTRACT

Differentiation-therapy is an important approach in the treatment of cancer, as in the case of erythroid induction in chronic myelogenous leukemia. Moreover, an important therapeutic strategy for treating beta-thalassemia and sickle-cell anemia could be the use of drugs able to induce erythroid differentiation and fetal hemoglobin (HbF) accumulation: in fact, the increased production of this type of hemoglobin can reduce the clinical symptoms and the frequency of transfusions. An important class of erythroid differentiating compounds and HbF inducers is composed by DNA-binding chemotherapeutics: however, they are not used in most instances considering their possible devastating side effects. In this contest, we approached the study of erythrodifferentiating properties of furocoumarins. In fact, upon UV-A irradiation, they are able to covalently bind DNA. Thus, the erythrodifferentiation activity of some linear and angular furocoumarins was evaluated in the experimental K562 cellular model system. Quantitative real-time reverse transcription polymerase-chain reaction assay was employed to evaluate the accumulation of different globin mRNAs. The results demonstrated that both linear and angular furocoumarins are strong inducers of erythroid differentiation of K562 cells. From a preliminary screening, we selected the most active compounds and investigated the role of DNA photodamage in their erythroid inducing activity and mechanism of action. Moreover, some cytofluorimetric experiments were carried out to better study cell cycle modifications and the mitochondrial involvement. A further development of the work was carried out studying the erythroid differentiation of photolysis products of these molecules. 5,5′-Dimethylpsoralen photoproducts induced an important increase in γ-globin gene transcription in K562 cells.

1. Introduction

Furocoumarins are well known natural or synthetic compounds, which derive from a linear (psoralens) or angular (angelicins) condensation of a coumarin with a furan ring. Some of them are employed in PUVA (Psoralen + UVA) therapy for the treatment of autoimmune or hyper-proliferative skin diseases, including psoriasis and vitiligo. PUVA therapy efficacy is due to a combination of psoralen administration and UV-A irradiation. In fact, when activated by UV-A light, furocoumarins induce many biological effects, such as photocycloadditions to DNA, immune system modulation, reactions with proteins, RNA and lipids [1]. Thanks to the development of the photopheresis, the PUVA therapy has amplified its application to some specific tumor forms such as cutaneous T-cell lymphoma [2].

Although the first furocoumarin was introduced in clinical practice as early as 1974 [3], these molecules still draw the attention of the scientific community. In fact, many new potential therapeutic applications for furocoumarins are found. For instance, some psoralen derivatives, such as 8-methoxypsoralen, showed anticonvulsant properties [4]; 4,6,4′-trimethylangelicin demonstrated to be potentially useful in the treatment of cystic fibrosis thanks to its anti-inflammatory activity and its potentiating action on the CFTR membrane channel whose dysfunction causes that disease [5]. Moreover, furocoumarins were found to induce various processes of differentiation. Psoralen is able to stimulate osteoblast differentiation without irradiation as demonstrated by Tang et al. [6],

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while with or without light activation, many furocoumarins induce erythroid differentiation in different cellular models [7–9]. This latter property can be useful for the treatment of hematologic diseases, such as β-thalassemia: at present, an important therapeutic strategy is the administration of fetal hemoglobin (Hb) inducers to reduce clinical symptoms and blood transfusion requirement [10].

The aim of our study was to evaluate the activity of six linear and five angular furocoumarins on the induction of erythroid differentiation expression of globin genes in the human leukemia cell line K562. These molecules were not fully checked for their potential erythro-differentiation so far. The K562 cell line, isolated from a patient with chronic myelogenous leukemia in blast crisis, is often used as *in vitro* experimental system for the first screening of new fetal Hb inducers [11]. The K562 cell line presents a low amount of Hb-synthesizing cells under standard cell-growth conditions. After the treatment with suitable inducing compounds, massive erythroid induction occurs, with a clear increase in the expression of human α and γ globin genes and a cytoplasmic accumulation of Hb Portland ($\zeta_2\gamma_2$) and Hb Gower 1 ($\zeta_2\varepsilon_2$) [10,12,13].

We then focused our attention on the most active compounds in order to obtain preliminary data about their mechanism of action and about the mode of cellular death induced by them.

A second part of our study was related to the well established observation that after UV-A irradiation, psoralens undergo photolysis with the formation of new species in solution, the so called photooxidation photoproducts (POPs). POPs also present some biological activity: in fact, some papers showed their antileukemic and immunosuppressive effects, which led us to hypothesize their possible biological contribution in PUVA therapy [14,15]. Recently, we also isolated and reported the erythroid differentiation induction by a specific 5-methoxypsoralen photoproduct [16].

Thus, the effect of POPs was also evaluated on the expression of embryo-fetal globin genes in K562 cells by quantititative real-time reverse transcription polymerase-chain reaction assay (RT-qPCR).

2. Materials and methods

2.1. Chemicals and cellular buffers and media

Psoralens and angelicins belong to the collection of the Sciences of Drug Department in Padova University [17–19]. If not specified elsewhere, all chemicals, biological buffers and cellular media were purchased from Sigma–Aldrich.

2.2. Irradiation procedure

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

2.3. Cell culture

The human leukemia K562 cells were cultured in a humidified atmosphere of 5% CO_2/air in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS; Invitrogen), 100 units/mL penicillin and 100 mg/mL streptomycin.

2.3.1. Phototoxicity and benzidine test

Suspensions of 30,000 K562 cells/mL in complete medium were seeded in individual wells of a 24-well tissue culture microtiter plate. The plates were incubated at 37 °C for 24 h prior to the experiments. Stock solutions of furocoumarin derivatives were prepared in methanol and then diluted with Hank's balanced salt solution (HBSS pH 7.2; the concentration of methanol was always lower than 0.5%) for irradiation experiments. After medium removal, 1 mL of the drug solution was added to each well, incubated at 37 °C for 30 min and then irradiated (1 and 2 J/cm^2, which correspond to 4 and 8 min of irradiation at 0.25 J/cm^2). After irradiation, the solution was replaced with complete medium and the plates were incubated for 5–7 days. The medium was never changed during this period. Erythroid differentiation was determined by counting blue benzidine-positive cells after suspending the cells in a solution containing 0.2% benzidine in 10% H_2O_2 and 0.5 M glacial acetic acid [7]. Cell phototoxicity was assessed by the MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)] test 5 days after irradiation [20]. The irreversibility of erythroid differentiation was also assessed: 6 days after irradiation, 10,000 cells were plated in a new plate with fresh medium and after further 4 days, they were counted after benzidine-staining as elsewhere described [21].

2.3.2. Cell cycle analysis

1×10^6 K562 cells were incubated for 24 h and then irradiated (1 J/cm^2) in HBSS with or without the test compounds. After 24 h from irradiation, cells were fixed with ice-cooled ethanol (70% v/v), treated overnight with RNAse A (0.1 mg/mL) in phosphate saline buffer and finally stained with propidium iodide (PI, 0.1 mg/mL). Samples were analyzed on a BD FACS Calibur flow cytometer collecting 10,000 events. Results of cell-cycle analysis were examined using WinMDI 2.9 (Windows Multiple Document Interface for Flow Cytometry) [20].

2.3.3. Mitochondrial dysfunction

K562 cells (300,000 cells/mL) were seeded in 24-well microplate and incubated for 24 h prior irradiation. After medium removal, 1 mL of the drug solution was added to each well, incubated at 37 °C for 30 min and then irradiated (1 J/cm^2). After irradiation, the solution was replaced with complete medium and the plates were incubated for 24 h. Cells were collected by centrifugation and re-suspended in 1 μM JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine) solution in HBSS or in 100 nM NAO (10-*N*-nonyl acridine orange) solution in RPMI medium. The cytofluorimetric analysis (BD FACS Calibur flow cytometer) was performed collecting green (FL1) and orange (FL2) fluorescence for JC-1 staining and only the green one (FL1) for NAO staining in at least 10,000 events for each sample [22,23].

2.4. Preparation of photoproducts

Solutions of derivatives in methanol were irradiated in a quartz cuvette with different UV-A doses (0, 8, 16 and 32 J/cm^2). After the irradiation, the solution was lyophilized, suspended in a known volume of methanol and stored at −20 °C. Concentrations of unknown photoproduct mixtures in this paper were expressed as if the initial psoralen was not photodegraded.

2.5. mRNA measurements

RNA was isolated from K562 cells and measured by reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) as described [24] using gene-specific double fluorescence labeled probes in an ABI Prism 7700 Sequence Detection System version 1.7.3 (Applied Biosystems). The following primer and probe sequences were used: α-globin forward primer, 5′-CAC GCG CAC AAG CTT CG-3′; α-globin reverse primer, 5′-AGG GTC ACC AGC AGG CAG T-3′; α-globin probe, 5′-FAM-TGG ACC CGG TCA ACT TCA AGC TCC T-TAMRA-3′; 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