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Structure and Biological Activity of Furocoumarins

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Abstract In this review we summarize the structure and biological effects of linear and angular psoralens. These compounds exhibit very interesting biological effects on cell cycle, apoptosis and differentiation. These molecules should be considered as promising drugs in the therapy of several diseases, including psoriasis, mycosis fungoides, cancer. In addition, pre-clinical data demonstrate a possible employment of these molecules for the treatment of β-thalassemia.

Keywords Apoptosis · Cell cycle · Erythroid differentiation · Erythroid precursors · K562 cells · Psoralens

Abbreviations
5-MOP 5-methoxypsoralen
8-MOP 8-methoxypsoralen

PUVA psoralens plus ultraviolet A
MF mycosis fungoides
PI propidium iodide
FACS fluorescence activated cell sorter
PS phophatidylserine

1 Introduction

Psoralens, also known as furocoumarins, are naturally occurring or synthetic tricyclic aromatic compounds and are derived from the condensation of a coumarin nucleus with a furan ring [1–10]. Several new furocoumarins have been isolated from natural sources [10, 11]. In addition, the synthetic methods for production of these molecules have been described and reviewed [10]. The synthetic methods can be organized on the basis of the key step used for the formation of the two different oxygenated rings. In this respect, there are three possibilities: (i) formation of the furan ring onto the coumarin, (ii) formation of the pyrone ring onto the benzofuran and (iii) the simultaneous formation of both oxygenated rings onto a benzene unit. Psoralens have been extensively studied and demonstrated to retain interesting biological effects on eukaryotic cells, allowing biomedical applications and the development of clinical trials [12, 13, 20]. The biological importance of furocoumarins mainly focuses on their relevant applications in photochemotherapy, as pointed out in several reviews [21–23].

2 Linear Psoralens

Linear psoralens widely used in therapy are 5-methoxypsoralen (5-MOP or bergapten) and 8-methoxypsoralen (8-MOP) (see Fig. 1) [24–29]. In addition, several analogues have been described [25, 26]. It is generally accepted that these molecules cause cell damage by covalent binding to DNA following UVA irradiation; they in fact exhibit a planar tricyclic structure with two photoreactive sites (3,4-pyrone and 4′,5′-furan double bonds). The initial intercalation and interaction with double stranded DNA is not characterized by covalent bonds, but, upon absorption of a photon of UVA, a pyrimidine residue (preferentially a thymine) of the DNA covalently binds to the first photoreactive site with a 5,6-double bond. The resulting monoadduct can form a diadduct by absorbing a second photon, if a new pyrimidine on the opposite strand of DNA is available for an interstrand cross-link. In conclusion, the planar structure of psoralens helps them to intercalate between nucleic acid base pairs. UVA irradiation activates the intercalated complex, result-

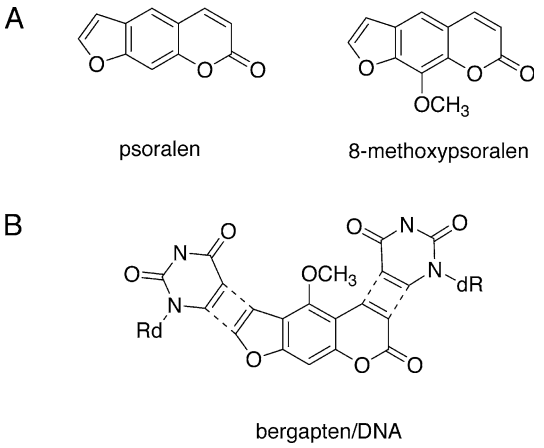

Fig. 1 **A** Structure of linear psoralens. **B** Complex between bergapten and DNA

ing in the formation of photoadducts with pyrimidines in cellular DNA. 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), that are believed to be the primary cause of photoinduced cell killing [1].

The crosslinking process depends on the structure of the psoralen derivative. Linear furocoumarins form crosslinks efficiently with a yield up to 50% of the overall adducts formed.

Substitution of the psoralen molecule by alkyl groups, like methyl(s), which increase lipophilicity of the compound and, within certain limits also the intercalation capacity, is likely to modify this picture. In particular, the photoreaction of 4-methyl psoralen derivatives with DNA yields a lower percentage of pyrone-side monoadducts. This may be rationalized in term of hindering effect due partly to the methyl group of thymine, although the cross-linking capacity is not affected. The photoreactivity of one of the double bonds of the furan and pyrone moieties may be reduced or completely eliminated if the substituting groups are bulkier or exhibit electron-withdrawing properties. Also this could be achieved by introducing a fourth aromatic ring, fused at the 4′,5′ or 3,4 position. As a result, linear monofunctional compounds, including carbethoxy-, pyrido-benzopsoralen and allopsoralen, were developed [11, 25].

2.1 Biological Activity of Linear Psoralens

Nowadays, many human skin diseases, such as psoriasis, T-cell lymphoma (cutaneous T-cell lymphoma, CTCL; mycosis fungoides MF), and vitiligo, are commonly treated with a combination of psoralens and UVA radiation commonly referred as PUVA (psoralens plus UVA) therapy.

Psoriasis

As far as treatment of psoriasis, PUVA has been compared in several review articles with the current practice of phototherapy with ultraviolet (UV) radiation without sensitizers [18–20]. Both treatment modalities are well established in therapy of psoriasis. Phototherapeutic regimens use repeated controlled UV exposures to alter cutaneous biology, aiming to induce remission of skin disease. Although UVB has been used for a longer time than PUVA, the latter has been evaluated and validated in a more detailed and coordinated fashion [18]. It is widely accepted that lesion clearance is obtained with oral psoralens (such as 5- or 8-methoxypsoralen), plus UVA exposure in patients with vitiligo or psoriasis, although differences were found in respect to the total UV exposure needed to obtain clinical outcomes [20]. Interestingly, differences among the employed psoralens were described. For instance, the incidence and severity of adverse events was generally lower in PUVA 5-MOP than in PUVA 8-MOP recipients. Nausea and/or vomiting, pruritus and erythema were the most commonly reported adverse events in the short term; they occurred about 2 to 11 times more frequently in 8-MOP than 5-MOP recipients. Adverse hepatic events after oral administration of the drug were uncommon. By contrast, long term tolerability data for PUVA are still scarce; however, carcinogenicity was not reported during a 14-year observation period of 413 patients with psoriasis [20].

T-cell Lymphoma

Linear psoralens have been extensively studied in the treatment of T-cell lymphoma (mycosis fungoides, MF) [22, 30–35]. Treatment of MF is indicated to reduce symptoms, improve clinical appearance, prevent secondary complications, and prevent progression of disease, all of which may have an impact on survival. It has been reported that psoralen and ultraviolet A radiation is effective in early-stage MF, inducing complete remissions in most patients. Psoralens and ultraviolet A radiation may also be combined with low doses of interferon (IFN)-alpha to treat stage I/II disease. Extracorporeal photopheresis may also be used successfully, but it is not generally available [31].

3 Angular Psoralens

Among psoralens-related compounds, the angular angelicin-like isomers (the structure of angelicin is shown in Fig. 2A) are both natural or synthetic compounds, present for instance in the medicinal plant *Angelica arcangelica*, that could exhibit interesting pharmacological activity when compared with linear psoralens, including low toxicity and low DNA-binding activity. Unlike

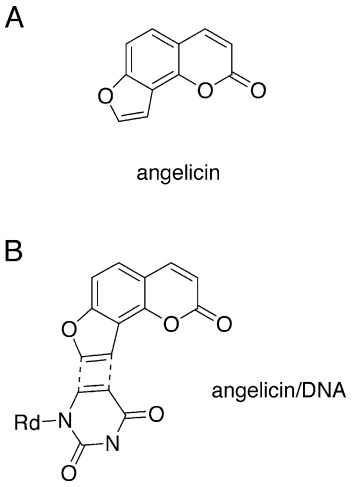

Fig. 2 Structure of angelicin (**A**) and its interaction with DNA (**B**)

linear psoralens, angelicin and its angular analogues, are monofunctional isopsoralen isomers and cannot create interstrand cross-links because of their angular geometric structure [36, 37] (see Fig. 2). In conclusion, these angular psoralen derivatives allow only monofunctional photobinding, thus reducing undesirable side effects, such as genotoxicity and risk of skin cancer.

3.1
Biological Activity of Angular Psoralens

These molecules might be of great interest to induce biological functions without (or with limited) toxicity. Recently, some of us have reported the accumulation of γ-globin mRNA in human erythroid cell treated with angelicin in the absence of UVA irradiation [38, 39]. Angelicin was able to stimulate increase of γ-globin mRNA and fetal hemoglobin (HbF) production. This feature is of potential clinical relevance. Psoralens could be proposed for the therapy of β-thalassemia and sickle-cell disease [39–46]. Indeed, even a minor increase in the production of HbF by patients affected by these diseases has been described to be beneficial. Patients treated with HbF inducers have been reported to be converted to transfusion-independent individuals [39].

4
Psoralens and the Cell Cycle

For flow cytometric analysis of DNA content, cells under investigation, in the exponential phase of cell growth, can be treated at different concentrations

with psoralens. After an incubation period that can be varied at the investigator's will, the cells are centrifuged, fixed in ice-cold ethanol, then treated with lysis buffer containing RNAseA, and finally stained with propidium iodide (PI). Samples can be analyzed using standard fluorescence-activated cell sorters (FACS, such as the Becton Coulter Epics XL-MCL flow cytometer). This and similar approaches give evidence for psoralen-dependent alteration of the cell cycle. In fact, flow cytometric analysis of DNA content indicate that treatment of eukaryotic cells with increasing concentrations of both linear and angular psoralens, is associated with deep changes of cell cycle profile. For example, many papers showed that irradiation of lymphocytes, lymphoblastoid cell lines and human fibroblast in the presence of psoralens induce a G2/M arrest of the cell cycle [28, 47–50]. In addition, Jorges et al. showed that in a human keratinocyte cell line (HaCat) 8-MOP induces a cell cycle arrest in S-phase. This block may be abrogated by treating the cells with caffeine, a well know inhibitor of the ATM (ataxia-telangiectasia-mutated) and ATR (ATM and Rad3 related) protein kinases, two key enzymes involved in the down stream cellular response to DNA damage. In a recent paper, Viola et al. [50] demonstrated a differential response of linear and angular derivatives of psoralens in a human promyelocytic cell line (HL-60). They studied two linear (5-MOP, 8-MOP) and two angular derivatives (angelicin and trimethylangelicin) and the results obtained shown that psoralen derivatives efficiently induce apoptosis, as the two angular compounds are the most potent and caspase-3 is essential for this process. In the case of linear derivatives this event is preceded by a cell cycle block in G2/M phase require depending on different mechanisms involved in the phototoxicity of psoralen derivatives.

5
Psoralens and Apoptosis

Apoptosis can be analysed by several approaches, including immunohistochemistry analysis of DNA fragmentation, Annexin release assay, detection of Sub-G1 population in FACS analysis, increase of caspase activity. For instance, after treatment for 5 days with 5-MOP or angelicin, the cells can be rinsed two times with a PBS solution, fixed in paraformaldehyde at room temperature and apoptotic cells can be detected by several reagent kits, such as the DeadEnd Colorimetric Apoptosis Detection System (Promega). Measurement of apoptosis is calculated as the % of apoptotic nuclei (dark brown nuclei) versus total nuclei, as shown in the representative experiment of Fig. 3b. A dark brown signal indicates positive staining, while shades of blue-green to greenish tan indicate a non-reactive cell. The data obtained indicate that both linear and angular psoralens (5-MOP and angelicin in the representative experiment reported) induce high level of apoptosis, even without UVA

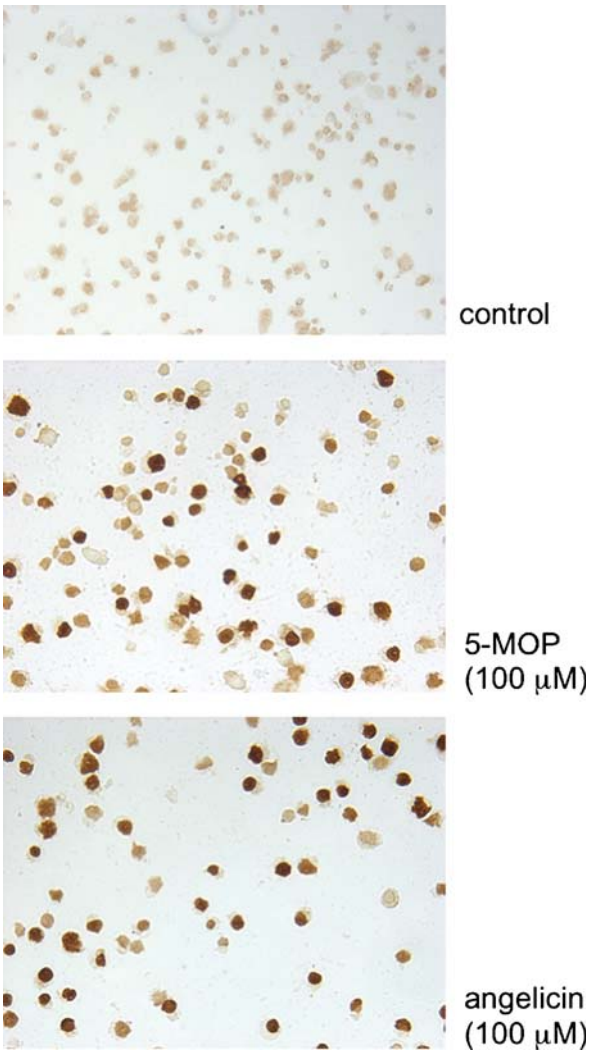

Fig. 3 Induction of apoptosis of human leukemic K562 cells treated for 5 days with 5-MOP and angelicin, as indicated

treatment of the cells. To characterize psoralen-induced apoptosis, biparametric cytofluorimetric analysis can be also performed using propidium iodide (PI) and Annexin-V-FITC, which stain DNA and phosphatidylserine (PS) residues, respectively. Annexin-V is, indeed, a Ca^{2+} dependent phospholipid binding protein with high affinity for PS. This assay represents a measurement of apoptosis, because externalization of PS occurs in the early stages of the apoptotic process. Later, analysis of the cell cycle might identify apoptotic cells as a sub-G_1 peak, evidence of the DNA fragmentation occurring

at later stages of apoptosis. The data available in the literature and our own concurrently indicate that most of linear and angular psoralens activate the apoptotic pathway in a concentration-dependent manner [51–57].

6 Psoralens and the Therapy of β-Thalassemia

For assessing the activity of linear and angular psoralens on human erythroid cells, the experimental system considered to be more reliable is constituted by erythroid precursor cells isolated from normal donor or patients affected by β-thalassemia. The employed two-phase liquid culture procedure has been previously described [46]. Mononuclear cells are isolated from peripheral blood samples of normal donors by Ficoll–Hypaque density gradient centrifugation and seeded in α-minimal essential medium supplemented with fetal bovine serum (FBS), cyclosporine A, and conditioned medium from the 5637 bladder carcinoma cell line [46]. The cultures are 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 non-adherent cells are harvested, washed, and then cultured in fresh medium containing human recombinant erythropoietin (EPO). This part of the culture is referred to as phase II. Psoralens are added on day 6 of phase II. Cell samples are analyzed on day 9 of phase II. Quantitative real-time PCR assay of gamma-globin and alpha-globin transcripts can be carried out using gene-specific double fluorescently labeled probes in a 7700 Sequence Detection System version 1.7.1 (Applied

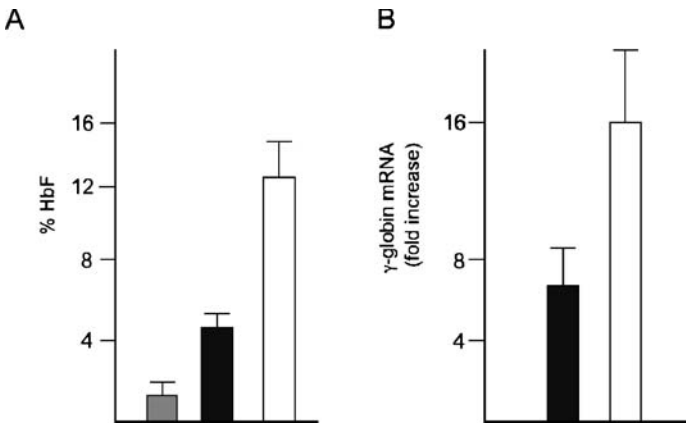

Fig. 4 Effects of angelicin (*white boxes*) on HbF production (**A**) and γ-globin mRNA expression (**B**). HbF was analysed by HPLC, γ-globin mRNA accumulation by real-time quantitative RT-PCR. The effects of angelicin were compared to those of hydroxyurea (*black boxes*; *grey box* = control untreated cells)

Biosystems, Warrington Cheshire, UK). The results obtained indicate that psoralens should be included in the class of HbF inducers, as shown in Fig. 4, which demonstrates high level of HbF induction of HbF by angelicin, quantified by HPLC (High Performance Liquid Chromatography).

7 Conclusion and Future Perspectives

The conclusion of this review is that linear and angular psoralens [1–11, 58, 59] exhibit very interesting biological effects on eukaryotic cells and should be considered as promising drugs in the therapy of several diseases, including psoriasis, mycosis fungoides, cancer.

Of great interest is the possibility that this class of molecules could be a source of lead compounds. In fact, it has been recently shown that some products of photolysis of psoralens may exhibit interesting biological effects in the dark. In this context, Potapenko et al. suggested that the photobiological activity of psoralens derives from their photooxidized products formed during pre-irradiation (POP) [60]. This author in fact analyzed the efficacy of crude pre-irradiated solutions of psoralens in a variety of biological models [61–64]. First, they proved that the solutions are active only when irradiation is carried out in the presence of oxygen and concluded that the activity should be ascribed to photooxidized psoralen species (POP). It was found that POP induces hemolysis of erythrocytes [65], modifies the "respiratory burst" of phagocytes and increases the permeability of their membrane [66]. Chemically oxidized or photooxidized psoralens inhibit chemotactic activity of polymorphonucleated cells and induce mutagenic and lethal effects in microorganism. The products of photooxidation of psoralens administered to mice have been shown to induce modulation of the T-cell mediated immune response and inhibit growth of grafted EL-4 lymphoma [67]. Recently, Caffieri et al. [68] isolated from a preirradiated solution of psoralens three cytotoxic molecules. These compounds induce apoptosis in a lymphoblastoid cell line that was preceded by mitochondrial dysfunction caused by the opening of the mitochondrial transition pore. Whether these molecules are involved in the mechanism of PUVA action remains to be clarified.

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