Growth inhibition of mammalian cells by synthetic and natural photosensitising agents

A.M. Christodouloupoulos, J.J. Osman and H.C. Lynch*

*Biotechnology and Biochemical Engineering Laboratories, School of Food Biosciences, Whiteknights, P.O. BOX 226, RG6 6AP Reading, United Kingdom; *Author for correspondence (e-mail: H.C.Lynch@reading.ac.uk; phone: 0044 01189 318593; fax: 0044 01189 318000)*

Received 14 November 2001; revised 27 November 2001; accepted in revised form 23 September 2002

Key words: Furocoumarins, Growth cycle, Growth inhibition, Psoralen, UV-A exposure

Abstract

A mammalian cell line, J774, was susceptible to both synthetic and natural photosensitising agents after irradiation with long-wave ultraviolet light. Both UV-A light and psoralen did not affect cell growth individually; a reduction in visual confluency was achieved only when psoralen and UV-A light were used in combination. The maximum visual confluency decreased by 55% when 50 ppm psoralen was added to a growing culture and irradiated with UV light for 3 min. Decreasing the UV-A exposure times from 3 min to 3 s did not greatly affect the maximum total visual confluence reached using different synthetic psoralen concentrations, but did affect the rate at which cell death occurred. The 3 min exposure time resulted in a rapid decrease in cell numbers in comparison to 3 s exposure time. Synthetic psoralen was found to have an increasing photosensitising activity with increasing concentration using a logarithmic shift between 0.5 ppm and 50 ppm. A visual confluency of 45 % was achieved using concentrations of 50 ppm psoralen, and 70% visual confluency using 0.5 ppm. Natural mixtures of furanocoumarins containing psoralens, obtained from two separate parsley sources, were found to have greater efficacy at inhibiting the growth cycle of the cells when compared to the synthetic psoralen.

Introduction

Linear furocoumarins are plant metabolites that belong to the phytochemical family of coumarins and these compounds occur predominantly in three plant families, the Umbelliferae, the Rutaceae and the Solanaceae. Plants from the Umbelliferae family include parsley, angelica herb and parsnip, and all contain high concentrations of linear furocoumarins (Beier et al. 1994; Ceska et al. 1987). Linear furocoumarins are benz-2-pyrone derivatives with a furan ring at the 6,7 position. These compounds have phototoxic activity and are thought to be produced as part of the plants defence mechanism against various environmental stresses, such as fungi (Chappell and Hahlbrock 1984).

The inhibitory effect of linear furocoumarins against microorganisms and especially bacteria has been extensively studied. Psoralen, the structurally

simplest member of the chemical group, has been shown to have phototoxic action against *E. coli* 0157:H7, *Listeria*, *Erwinia* and *Micrococcus* strains (Manderfeld et al. 1997; Ulate-Rodriguez et al. 1997; Hashem and Sahab 1999). Linear furocoumarins have also been reported to be fungitoxic (Beier et al. 1983; Downum 1992; Hashem and Sahab 1999), shown toxic activity against insects (Beier et al. 1983; Berenbaum et al. 1991) and inhibit the growth of plants (Downum 1992). Ashwood-Smith et al. (1980) also demonstrated the lethal effects of 5-methoxypsoralen (5-MOP), used in some suntan preparations, on Chinese Hamster Ovary cells when irradiated with UV-A light.

The biological activity of psoralen and other linear furocoumarins against bacteria, fungi, mammalian cells and plant species has been attributed mainly to their photoreactivity towards the pyrimidine bases of DNA. Under irradiation in the ultraviolet region the

intercalated psoralen molecule can form covalent bonds with pyrimidine bases. Psoralen can form either monoadducts, leading to harmful effects, or bifunctional adducts, which leads to extensive damage and lethal effects to cellular components (Grekin and Epstein 1981). The result is inhibition of DNA replication and transcription (Cimino et al. 1985; Song and Tapley 1979).

Despite their toxic activity against a range of biological life, psoralen, in conjunction with UV-A irradiation, have been used for clinical treatment of dermatological disorders since the 1970's. The treatment, known as PUVA therapy, was first described by Parish et al. (1974) and since then has been established as a clinical therapy for psoriasis and vitiligo. In addition psoralen is currently being used in clinical trials for the decontamination of transfusion products, such as platelet concentrates and blood plasma (Coghlan 1997; Lin et al. 1994).

The lethal effects of linear furocoumarins on mammalian cells, when used in combination with UV-A light, has been recognised as a possible treatment for the destruction of tumor cells *in-vivo*. Localised use of psoralen at the tumor site, combined with surgical UV-A probes to irradiate the area has possibilities for the treatment of, for example, breast cancer. Despite widespread recognition of the opportunities available for the use of the combined treatment, little work has been conducted on determining the inhibitory effects of psoralen and other linear furocoumarins with UV-A light on growing cultures of mammalian cells. This study aims to provide growth curve data on a mammalian cell line to demonstrate the lethal effects of both a natural mixture of linear furocoumarins from parsley extracts and synthetic psoralen when used in combination with UV-A light. The study aims to demonstrate the effect of both psoralen concentration and UV-A exposure time on cell viability.

Methods and Materials

Animal Cell Line and Culture Conditions

The cell line J774, from mouse macrophage cells, was used in all experiments and is anchorage dependent. Cells were grown in a low glucose variety of Dulbecco's modification of Eagle's Minimum Essential Medium (DMEM) with Foetal Bovine Serum (FBS). 88% DMEM was added into a 75 cm² flask together with 10% FBS and 1% L-glutamine. 1% penicillin

and streptomycin was added to prevent contamination. Cells were cultured in a humidified incubator at 36.5 °C and gassed with a 5 % CO₂/air mixture to maintain the pH of the medium.

Experiments with psoralen were conducted in multiwell plates (6 well Corning Cloning plates) to allow numerous samples to be processed. The wells were filled with 2 ml of medium with a cell seeding density of 1×10^5 cells/ml. After the cells were deemed to be entering the exponential phase, the psoralen and UV-A were administered.

Maintenance of Cell Culture

Cells were sub-cultured at a ratio of 1:8. The sub-culturing was achieved through discarding the medium, and adding 1 ml of warm trypsin/EDTA (Sigma) to the side of the flask. The cells were rinsed with the trypsin/EDTA, then the rinse discarded. A further 2 mls of trypsin/EDTA was added again to the side of the flask and then incubated for 20 mins with some gentle taping to remove the attached cells. The trypsin was subsequently inactivated by adding 8 mls of warm medium containing FBS. The cells were dispersed by repeated pipetting and the mixture was added to a 50 ml centrifuge tube and centrifuged at 900 rpm (or 146 g) for 5 min. The supernatant was discarded, leaving the cells. 10 ml of warm medium was added and pipetted gently up and down to re-suspend the cells. A 100 µl sample was taken for cell counting. The remaining cells were diluted to the appropriate seeding concentration

Visual Confluency and Cell Counting

Visual confluency was measured as the percentage of coverage of each well with monolayer of cells, using a microscope to examine the coverage. Three wells were used for each time point and measured using two independent observers. Error bars are shown on the graphs for the data obtained. A classic cell growth curve was obtained using the visual confluency method for untreated cells.

Visual confluency was used as the measurement technique due to the adherent nature of the cells, resulting in the formation of clumps when suspended using trypsin. The cell clumps made impossible the use of cell counting for cell viability numbers. Visual confluency was measured immediately on removal from the incubator and within a 5 min period to prevent a pH rise affecting cell viability.

Synthetic Psoralen Addition

10 mg of synthetic psoralen (Sigma) was dissolved in 2 ml of ethanol and made up to 50 ml with pure water, giving a stock solution of 200 ppm (200 mg l^{-1}). The stock solution was stored at 5°C and diluted in water when required to give concentrations of 50 ppm (50 mg l^{-1}), 5 ppm (5 mg l^{-1}) and 0.5 ppm (0.5 mg l^{-1}). Multiwell plates containing 2 ml of culture medium were seeded with 1×10^5 cells/ml and allowed to grow to exponential phase after approximately 2 d. On entering exponential phase 100 μl of either 0.5 ppm, 5 ppm or 50 ppm synthetic psoralen was added, allowed to rest for 10 min then subjected to UV-A light for three minutes. The controls used were operated under identical growth conditions using alcohol in place of the psoralen and contained either a) no psoralen and UV-A light, b) 100 μl of 50 ppm psoralen without UV-A light, and c) UV-A light for three minutes without psoralen added.

Dried Parsley Extracts

Parsley (*Petroselinum crispum*) extracts were obtained by the following procedure. Psoralens were extracted three times with ethyl acetate from 10 g of parsley. The extract was filtered through filter paper and the solvent evaporated under constant supply of nitrogen in a water bath at 35°C until completely dry. The filtrate was then dissolved in 1 ml of HPLC grade ethanol and supplemented with distilled water to give a volume of 8 ml. The glycosidated bonds were hydrolysed by taking 4 ml and adding 1 ml of 2 M HCL. The sample was neutralised with 1 M NaOH. The extracts were quantified by reverse phase HPLC using synthetic psoralen, xanthotoxin and bergapten standards (Sigma). The parsley sample sources were obtained from Bart Spices, Bristol (sample A), and from a Sainsbury's (sample B). Sample A analysed by HPLC contained 0.6 ppm psoralen, 4.6 ppm bergapten and 0.7 ppm xanthotoxin. Sample B contained 2.1 ppm psoralen and 2.3 ppm xanthotoxin.

Natural Linear Furocoumarin Addition

Experiments using natural furocoumarins were treated in a similar fashion to those treated with synthetic psoralen. Cells were grown to exponential phase followed by addition of 100 μl of natural

furocoumarins. To the control samples was added the equivalent volume of alcohol.

Ultraviolet-A (UV-A) illumination Treatment

Cultures treated with 100 μl of the natural furocoumarin mixture or synthetic psoralen were allowed to rest for 10 mins in the incubator before UV-A light irradiation, to allow the psoralen to diffuse into the cell. Appropriate control samples containing the equivalent quantity of alcohol were also allowed to rest in the incubator before UV-A treatment. The UV-A light was administered using a Blak-Ray (model B100 AP) 100 watts high intensity ultraviolet lamp at a distance of 10 cm from the sample. During experiments conducted on the cell line, cultures were taken from the incubator and UV-A irradiated for the required amount of time before returning to the incubator.

Reproducibility

Growth curve experiments were conducted in duplicate, and visual confluency reading measured in triplicate for each time point by two independent observers. Error bars, which represent the error limits on the triplicate visual confluency readings, are shown on the graphs. Where error bars are not shown the standard error is less than 10%.

Results and Discussions

Effects of Psoralen and UV-A

Experiments were conducted to determine the effects of synthetic psoralen, UV light and a combination of the two on the growth curve of J774. Visual confluency was measured throughout the growth curve of the experimental wells and of each of the control wells. The results are shown in Figure 1.

The results show clearly that the three controls follow the general growth curve, whilst the cells with the psoralen and UV-A treatment have been inhibited. In each of the control experiments cell growth decreases after approximately 150 hr, presumably in response to either depletion of a growth limiting nutrient or build up of toxic by-product. However, where the cells were treated with psoralen/UV-A inhibition of cell growth occurred directly after addition of the psoralen and UV-A treatment, i.e. at mid

Figure 1. Growth curves of cultures treated with i) 50 ppm psoralen and 3 min UV-A light (■), ii) no psoralen or UV-A light (◆), iii) 50 ppm psoralen and no UV-A light (○) and iv) 3 min UV-A light and no psoralen (▲)

exponential phase. This immediate effect on cell growth was not seen in any of the control experiments at this time. These results suggest that the psoralen and UV-A treatment was responsible for the reduction in visual confluency, which reached 45% in samples treated with the combination of psoralen and UV-A light. Growth of the cells in the control experiments decreased from 100% visual confluency at the end of the exponential phase following either nutrient depletion or toxic build-up. The decrease at the end of the exponential phase in the control experiments was unrelated to the psoralen/UV-A treatment since all control experiments followed identical growth curves, including the control without psoralen, UV-A, or the combined treatment.

Psoralen and UV light as Variables of Cell Death

Psoralen and UV-A light combination clearly had a marked effect on the growth curve of J771. Both the concentration of psoralen and the exposure time of UV-A light are variables in the efficacy of the treatment. The following experiments were conducted to determine the effect of psoralen concentration and exposure time on cell death.

Varying Concentrations of Psoralen and UV-A exposure times

Three different concentrations, based on a logarithmic shift from 0.5 ppm to 50 ppm were used to determine the effect of psoralen concentration on cell death. 100

μl of psoralen at concentrations of 0.5 ppm, 5 ppm and 50 ppm were added to cultures and exposed to UV-A light for a fixed time of 3 min.

A clear difference is shown in Figure 2 between cultures where 0.5 ppm of psoralen was added and cultures where concentrations were above 5 ppm. The samples with 0.5 ppm added reached a visual confluence of approximately 72%, compared with 47% and 44% maximal visual confluency for 5 ppm and 50 ppm psoralen respectively.

To measure the effect of UV-A exposure on the cell line, the UV-A exposure time was shortened. Following psoralen addition at concentrations of 50 ppm, 5 ppm and 0.5 ppm, cells were exposed to UV-A light irradiation for 3 s. Figure 3 shows the growth curves of the cells for each of the psoralen concentrations. Once again concentrations of 0.5 ppm showed a lesser effect on cell death than that found at concentrations of 5 ppm and 50 ppm. The maximum visual confluency reached using 0.5 ppm psoralen with 3 s exposure time was approximately 70%, compared with visual confluency of 54% and 56% for psoralen concentrations of 5 ppm and 50 ppm respectively.

The UV-A exposure time of 3 mins provided a more rapid decrease in cell death when compared with exposure time of 3 s for each of the three concentrations of psoralen used. From the results in Figure 2,3 it would appear that UV-A exposure time does not affect the maximum visual confluency to a great degree, but does appear to affect the rate of cell death. Under conditions of 3 min exposure time cell numbers dropped rapidly from maximum to less than 20%

Figure 2. Growth curves of cultures treated with 0.5 ppm psoralen (▲), 5 ppm psoralen (■), 50 ppm psoralen (●) each with 3 min exposure of UV-A light, and the control with no psoralen or UV-A light (◆)

Figure 3. Growth curves of cultures treated with 0.5 ppm psoralen (▲), 5 ppm psoralen (■) and 50 ppm psoralen (●) each with 3 s exposure of UV-A light, and the control with no psoralen or UV-A light (◆)

within 50 hr, compared with 75 hr to 130 hr when exposure time of 3 s was used.

Natural Linear Furocoumarins from Parsley Extracts

Natural linear furocoumarins were extracted from parsley samples A and B, and used to determine the efficacy of the natural plant extracts on cell death in comparison with synthetic psoralen. 100 μ l of the parsley extract was added to cultures at mid exponential phase of growth and exposed to UV-A light for 3 min. The growth curves of cultures treated with 50

Figure 4. Growth curves of cultures treated with plant extracts from sample A (○), sample B (●) and synthetic psoralen at concentration 50 ppm (■) with 3 min exposure of UV-A light, and the control with no psoralen or UV-A light (◆)

ppm of psoralen, are shown in Figure 4, together with the growth curves of cultures treated with sample A and sample B extracts. From Figure 4 it can be seen that the growth of the cells treated with the natural plant extracts is clearly inhibited. Both growth curves treated with the natural extract achieve a similar maximum visual confluency to the cultures treated with 50 ppm synthetic psoralen. However, the rate of cell death was more rapid with the natural psoralens, and thus the extracted material appears to be more potent than the synthetic psoralen. The extract contained psoralen, as well as xanthotoxin, bergapten and possibly other furocoumarin photosensitising agents. The increased potency may be due to the combined effect of these different agents extracted, or the effect of one of the potent components in the extract.

Conclusions

Psoralen when used in combination with UV-A light showed a clear and rapid response in the death of the mammalian cell line J 771. Both the concentration and time of exposure of UV-A light had an effect on visual confluency reached and in the rate of cell death. In particular the increased exposure time from 3 s to 3 min at a psoralen concentration of 50 ppm had a very visible effect on the rate of cell death. Increasing the concentration from 0.5 ppm to 50 ppm have a visible effect on the visual confluency reached, especially with an exposure of 3 min was used.

Natural linear furocoumarins, obtained from dried parsley extracts, displayed a more toxic effect on the cell line than the synthetic psoralen. Cell death was more rapid with the natural extract and visual confluency reached similar level to the synthetic psoralen, despite having a lower concentration of psoralen in the extract. The reason for the increased toxicity with the natural extract may be due to the mixture of different furocoumarins in the extract. The parsley extract was shown to have 0.6 ppm psoralen, 4.6 ppm bergapten and 0.7 ppm xanthotoxin in sample A and 2.1 ppm psoralen and 2.3 ppm xanthotoxin in sample B. Xanthotoxin has previously been shown to have the most toxic effect against biological life in comparison with both bergapten and psoralen (Berenbaum et al. 1991). The combined effects of the psoralen, bergapten and xanthotoxin may have a more potent effect in the samples than if used individually. Berenbaum et al. (1991) also demonstrated the increased toxicity when a natural mixture of furocoumarins was used

against bacterial cells, when compared to an equimolar amount of xanthotoxin.

The increased potency of natural mixtures of furocoumarins may provide an effective treatment for the destruction of tumor cells *in-vivo* when used in combination with a surgical UV-A probe. Further study to find an effective mixture of natural psoralens is needed, together with the development of suitable UV-A probe that can be used in surgery.

References

- Ashwood-Smith M.J., Polton G.A., Barker M. and Mildenerger M. 1980. 5-Methoxypsoralen, an ingredient in several suntan preparation, has lethal, mutagenic and clastogenic properties. *Nature* 285: 407–409.
- Beier R.C., Ivie G.W. and Oertli E.H. 1983. Psoralens and other linear furocoumarins as phytoalexins in celery. *Phytochemistry* 22: 2595–2597.
- Beier R.C., Ivie G.W. and Oertli E.H. 1983. Psoralens as phytoalexins in food plants of the family Umbelliferae. *ACS Symposium Series* 234: 295–310.
- Beier R.C., Ivie G.W. and Oertli E.H. 1994. Linear furocoumarins and graveleone from the common herb parsley. *Phytochemistry* 36: 869–872.
- Berenbaum M.R., Nitao J.K. and Zangerl A.R. 1991. Adaptive significance of furanocoumarin diversity in *Pastinaca sativa*. *J Chem Ecol* 17: 207–215.
- Ceska O., Chaudhary S.K., Warrington P.J. and Ashwood-Smith M.J. 1987. Photoactive furocoumarins in fruits of some Umbellifers. *Phytochemistry* 26: 165–169.
- Chappell J. and Hahlbrock K. 1984. Transcription of plant defence genes in response to UV light or fungal elicitors. *Nature* 311: 76–78.
- Cimino G.D., Gamper H.B., Isacss S.T. and Hearst J.E. 1985. Psoralens as photoactive probes for nucleic acid structure and function. *Annu Rev Biochem* 54: 1151–1193.
- Coghlan A. 1997. Fruit and veg make for safer blood. *New Scientist* 7 June 1997. 20.
- Downum K.R. 1992. Light-Activated Plant Defence. *New Phytologist* 122: 401–420.
- Grekin D.A. and Epstein J.H. 1981. Psoralens, UVA (PUVA) and photocarcinogenesis. *Photochem Photobiol* 33: 957–960.
- Hashem F. and Sahab A.F. 1999. Chemical response of parsley and mentha herbs to certain stress agents. *Food Chemistry* 65: 29–33.
- Lin L., Londe H., Janda J.M., Hanson C.V. and Corash L. 1994. Photochemical inactivation of pathogenic bacteria in human platelet concentrates. *Blood* 83: 2698–2706.
- Manderfeld M.M., Schefer H.W., Davidson P.M. and Zottola E.A. 1997. Isolation and identification of antimicrobial furocoumarins in parsley. *J Food Prot* 60: 72–77.
- Parish J.A., Fitzpatrick T.B. and Tannenbaum L. 1974. Photochemotherapy of psoriasis with oral methoxsalen and long-wave ultraviolet light. *N. Engl. J. Med.* 291: 1207–1211.
- Song P.S. and Tapley K.J. 1979. Photochemistry and Photobiology of Psoralens. *Photochem Photobiol* 29: 1177–1197.
- Ulate-Rodriguez J., Schafer H.W., Zottola E.A. and Davidson P.M. 1997. Inhibition of *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Micrococcus luteus* by linear furanocoumarins in culture media. *J Food Prot* 60: 1046–1049.
- Ulate-Rodriguez J., Schafer H.W., Zottola E.A. and Davidson P.M. 1997. Inhibition of *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Micrococcus luteus* by linear furanocoumarins in a model food system. *J Food Prot* 60: 1050–1054.