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# Weekly Systemic Photodynamic Therapy with Aminolevulinic Acid Delays the Appearance of UV-Induced Skin Tumors in Hairless Mice

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Weekly Systemic Photodynamic Therapy with Aminolevulinic Acid Delays the Appearance of UV-Induced Skin Tumors in Hairless Mice

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**RÉSUMÉ**

La thérapie photodynamique (TPD) est une nouvelle modalité thérapeutique qui allie l'administration d'un photosensibilisateur, ou un précurseur de photosensibilisateur, à son activation subséquente par la lumière visible. L'activation par la lumière induit des processus photochimiques qui peuvent induire la destruction des tumeurs.

L'acide aminolevulinique (ALA) est un précurseur du photosensibilisateur protoporphyrin IX (PpIX). Après administration topique ou systémique d'ALA, la cellule produit et accumule la PpIX, ce qui la rend photosensible. La TPD avec ALA a été récemment approuvée par la « Food and Drug Administration » aux États-Unis pour le traitement des kératoses actiniques.

Dans le but de développer de nouvelles modalités de prévention pour les patients à haut risque de cancer de la peau, nous avons étudié l'effet de la thérapie photodynamique avec administration systémique d'ALA sur le développement des carcinomes spinocellulaires cutanés induits par les ultraviolets (UV) chez la souris. Des groupes de 20 souris ont été exposés tous les jours aux rayons UV générés par des tubes FS20, ainsi qu'une fois par semaine à la TPD après administration intra-péritonéale d'ALA suivie d'une exposition à la lumière visible 3 heures plus tard. Sept groupes contrôles ont aussi été étudiés: exposition à tous les jours aux UV et une fois par semaine à l'ALA seulement, exposition à tous les jours aux UV et une fois par semaine à la lumière visible seulement, exposition à tous les jours aux UV seulement, traitement

de TPD-ALA seulement, injection une fois par semaine d'ALA seulement, exposition une fois par semaine à la lumière visible, ou aucun traitement. Les souris ont été examinées à toutes les semaines pour la présence de tumeurs. Nous avons observé une différence significative dans la probabilité de survie sans tumeur pour les souris traitées une fois par semaine avec ALA-TPD comparativement aux autres groupes.

Le nombre total de tumeurs par souris à la fin de l'étude était inférieur dans le groupe traité avec la TPD-ALA. Le groupe traité à la TPD-ALA n'a pas montré d'augmentation de la mortalité ni d'incidence plus élevé de tumeurs larges. La spectroscopie de fluorescence in vivo a révélé que 3 heures après injection intra-péritonéale d'ALA, l'intensité de la fluorescence générée par la PpIX, était plus élevée au niveau de la peau normale qu'au niveau des tumeurs. Une analyse en microscopie de fluorescence quantitative a également montré que la quantité de PpIX était plus élevée au niveau de la peau normale par rapport aux tumeurs à 3 heures post-injection. En conclusion, un traitement ALA-TPD, une fois par semaine, a permis d'induire un délai dans l'apparition des tumeurs induites par les UV chez la souris sans poil malgré un niveau de PpIX dans les tumeurs inférieur à la peau non-tumorale adjacente.

**ABSTRACT**

Photodynamic therapy (PDT) is a new therapeutic modality, which combines the administration of a photosensitizer or a precursor of the photosensitizer and its subsequent activation by visible light. This light activation results in a sequence of photochemical processes that cause irreversible photodamage to tumor tissue.

Aminolevulinic acid (ALA) is a precursor of photosensitizer protoporphyrin IX (PpIX). After administration of topical or systemic ALA, the cells produce and accumulate PpIX, which is a highly photoactive molecule. ALA-PDT has recently been approved by the US Food and Drug Administration for the treatment of actinic keratoses.

In order to develop new preventive modalities for patients at higher risk for developing skin cancer, we studied the effect of photodynamic therapy with systemic administration of aminolevulinic acid on UV-induced skin cancer in the hairless mouse. Groups of 20 mice were exposed daily to UV radiation generated by FS20 tubes, and weekly to broadband visible light from a slide projector 3 hours after an intraperitoneal injection of ALA. Seven control groups were also treated with daily UV exposure and weekly ALA only, daily UV and weekly visible light only, daily UV only, weekly ALA-PDT only, weekly ALA, weekly visible light, or no treatment. Mice were examined weekly for the presence of tumors. We observed a significant difference in tumor free survival for mice treated weekly with ALA-PDT as compared to the other group.

In addition, the number of tumors per mouse at the end of the study was significantly lower in the UV-ALA-PDT group as compared to other groups. No increase in either mortality or appearance of large tumors was noted in the UV-ALA-PDT group. *In vivo* fluorescence spectroscopy revealed that at 3 hours after intraperitoneal injection of ALA, the protoporphyrin IX fluorescence intensity was higher in normal skin than skin tumors. The analysis by quantitative fluorescence microscopy confirmed that the quantity of PpIX was higher in normal skin as compare to tumors at 3 hours post-injection. In conclusion weekly systemic ALA-PDT delayed the appearance of UV-induced skin cancer in hairless mice despite the lower level of PpIX fluorescence in tumors as compared to adjacent skin at the time of light exposure.

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## ABBREVIATIONS

| | |
|---|---|
| NMSC | Non melanoma skin cancer |
| BCC | Basal cell carcinoma |
| SCC | Squamous cell carcinoma |
| Aks | Actinic keratoses |
| UVB | Ultra violet B |
| UVA | Ultra violet A |
| PDT | Photodynamic therapy |
| LDL | Low-density lipoprotein |
| HPD | Hematoporphyrin derivative |
| ALA | Aminolevulinic acid |
| BPD-MA | Benzoporphyrin derivative monoacid ring A |
| SnET2 | Tin ethyl etiopurpurin |
| PpIX | Protoporphyrin IX |
| IL-2 | Interleukin 2 |
| IL-1β | Interleukin 1β |
| TNF-α | Tissue necrosis factor α |
| IL-6 | Interleukin 6 |
| IL-10 | Interleukin 10 |
| IP | Intraperitoneal |

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# Chapter I

## Introduction

## 1.1 Skin cancer

Non melanoma skin cancer (NMSC), which includes basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), is the most common type of human cancer (Cohn, 1998; Strom and Yamamura, 1997). Squamous cell carcinoma arises from transformation of keratinocytes within the epidermis and has a tendency to metastasize (Marks, 1996). Actinic (solar) keratoses (Aks) and Bowen's disease (carcinoma in situ) are intraepithelial tumors, which are precursors of SCC (Sober and Burstein, 1995). Basal cell carcinoma, which rarely metastasizes, putatively arises from appendage cells located within the epidermis (Miller, 1995). Malignant melanoma is the third most frequent type of skin cancer. It arises from epidermal melanocytes, tends to metastasize fairly early and does not have a good prognosis when metastatic (Armstrong and Holman, 1987).

### 1.1.1 Epidemiology

#### 1.1.1.1 Incidence

There has been a recent increase in the incidence of skin cancer, especially in fair-skinned people (Armstrong and Kricker, 1996; Strom and Yamamura, 1997). NMSCs, which constitute more than one-third of all cancers in the United States, are the most common malignancies occurring in the Caucasian population each year (Boring *et al.*, 1991) with an estimated annual incidence in the United States of over 800,000 cases (Parker *et al.*, 1996). Basal cell carcinoma is by far the most common, accounting for about 80 percent of the NMSCs. Approximate number of new cases of NMSCs per year in Canada is 66,000 with 3% and 3.5% of an annual average increase of BCC and SCC respectively (Canada Cancer Info Databace).

#### 1.1.1.2 Mortality

The mortality due to NMSC is relatively low. Despite its rising incidence, NMSC mortality rates have decreased over the last few decades (Marks, 1995). Mortality from NMSC in the USA is 0.44 per $10^5$ per year (Weinstock *et al.*, 1991), with the main cause of death being metastases from squamous cell carcinoma.

### 1.1.2 Risk factor

The likelihood of developing skin cancer depends on constitutional predisposition (genotypic and phenotypic characteristics) as well as subsequent exposure to environmental risk factors. The major constitutional risk factor for skin cancer is skin color (Stern and Momtaz, 1984).

Epidemiologic studies suggest that chronic, repeated sun exposure is associated with the development of BCC and SCC (Koh, 1995). Most NMSCs occur on areas of the body that are commonly exposed to sunlight. In addition, the number of cases of NMSC increases proportionally with proximity to the equator, with the incidence of SCC doubling for each 8-10 degree of decline in latitude (Giles *et al.*, 1988).

Ultra violet B (UVB) radiation (290-320 nm) is the most carcinogenic waveband of the solar spectrum reaching the surface of the earth (Cole *et al.*, 1986). Tumor induction experiments in the hairless mouse model have shown that both UVB and Ultra violet A (UVA) can generate SCC (Berg *et al.*, 1993; de Laat *et al.*, 1997; de Laat and de Gruijl, 1996).

There are several risk factors for the development of SCC including sun exposure, systemic immunosuppression and chemical carcinogens. Immunocompromised patients have a higher risk of developing SCC. The incidence of SCC in renal transplant patients is 5- to 20-fold higher than that in the general population and increases by 5% per each post-transplantation year (Hardie and Strog, 1980). In the general population, BCC is four times more common than SCC. In renal transplant patients, however, the ratio is reversed with SCC being three times more common than BCC (Gupta *et al.*, 1986).

Chemical carcinogens that promote the development of NMSC (primarily SCC) include arsenic (Maloney, 1996), coal tar (Pion *et al.*, 1995), tobacco (Lindquist, 1979), and psoralens (Studniberg and Weller, 1993). Oral psoralens, which are potent photosensitizers, are used in conjunction with UVA light to treat psoriasis (i.e., PUVA therapy). PUVA therapy induces a dose-dependent increase in the risk of SCC (Stern and Laird, 1984).

Other environmental factors and medical conditions associated with an increased risk of NMSC (especially SCC) include ionizing radiation (Martin and Strong, 1970), chronic ulcers (Bostwick and Pendergrast, 1976), burn scars (Abbas and Beecham, 1988), various genetic syndromes (e.g., xeroderma pigmentosum (Kraemer *et al.*, 1987), albinism (Keller, 1963), epidermodysplasia verruciforsum (Ruiter, 1986), and the basal cell nevus syndrome (Nicholas and Soloman, 1966)), human papillomavirus infection (Moy and Elierzi, 1989), and a prior history of NMSC (Marghoob and Kopf, 1993; Schreiber *et al.*, 1990). Once an individual develops NMSCs, there is a 36-52% chance

that a new skin cancer will appear within 5 years (Marghoob and Kopf, 1993; Schreiber, *et al.*, 1990).

### 1.1.3 Prevention

As UV exposure is one of the major causes of NMSC, a decrease in the amount of UV from sunlight reaching the skin should decrease the risk of developing NMSC. Unfortunately, because skin cancer can take several years to develop, prospective studies looking at the influence of specific sun protection measures on the incidence of NMSC are difficult to perform. The current approach to prevent skin cancer focuses mainly on sun avoidance and sun protection. General recommendations for minimizing exposure to UV radiation include minimizing outdoor sun exposure, wearing physical protection (i.e. clothing, hat, and sunglasses) and using sunscreens.

Experiments in rodents and humans have shown that sunscreens can prevent UVB-induced skin cancers (Akin and Sayre, 1991; Elmets and Anderson, 1996; Kligman *et al.*, 1980; Walter and DeQuoy, 1980; Green *et al.*, 1999). However, certain retrospective epidemiological studies suggest that sunscreen use could be a risk factor for melanoma possibly because patients stay for longer periods under the sun when using sunscreens (Autier *et al.*, 1997; Autier *et al.*, 1995; Westerdahl *et al.*, 2000). Thus, it is important to emphasize that while sunscreens protect against sunburn, people should not use this protection to increase the time they spend under the sun.

There is currently a need for the development of alternative methods to prevent skin cancer. A method that would destroy microscopic skin cancers before they develop

into visible tumors could be a significant advance in skin cancer prevention. The ideal preventive agent would have few or no adverse effects, high efficacy against the most frequent types of skin cancer, low cost, and easy acceptability by the population at risk.

## 1.2 Photodynamic therapy

Photodynamic therapy (PDT) is one of the most promising new fields of medicine which involves the use of photosensitizing agents followed by light exposure in order to treat various diseases. This therapy results in a sequence of photochemical and photobiologic processes that cause irreversible photodamage to tumor tissue. The outcome of a PDT session depends on a number of variables including the photosensitizer used, the administration modality, the light source, and the treatment procedure.

The photosensitizing agent selectively accumulates in neoplastic cells (Rittenhouse-Diakun *et al.*, 1995; Van Hillegersberg *et al.*, 1992). Hence, there is preferential targeting of the cancer and sparing of surrounding tissues. Preferential localization of the photosensitizer to the tumor as opposed to adjacent normal tissue is important in order to achieve a high cure rate with minimal damage to normal tissue. The tissue selectivity of photosensitizers is poorly understood but seems to result from the complex interaction between the photosensitizer biochemical properties and modality of administration. In systemically administered photosensitizers, a higher lipophilicity has been associated with a greater selectivity for tumors (Kessel *et al.*, 1992). Photosensitizers including porfimer sodium and benzoporphyrin are transported by low-

density lipoproteins (LDL) in circulation, which can result in selective photosensitizer release in many types of tumor cells (Jori *et al.*, 1984). Some photosensetizers have weak or no affinity for LDL suggesting that another mechanism may be involved (Kessel, *et al.*, 1992).

Since most photosensitizing agents are fluorescent, drug localization can be determined by fluorescence microscopy and *in vivo* fluorescence spectroscopy (Andersson-Engels *et al.*, 1997; Berg and Harris, 1986). *In vivo* fluorescence spectroscopy allows the optimization of light exposure time for effective PDT. The *in vivo* pharmacokinetic measurement also helps to understand the mechanisms of localization of photosensitizers in neoplastic tissues, which, so far, are not well established. The knowledge of the drug localization in different tissue types and layers is obtained by investigating excised tissue samples by "quantitative" fluorescence microscopy.

PDT is a very effective treatment for actinic keratoses with reported cure rates as high as 100 % (Jeffes *et al.*, 1997; Kennedy *et al.*, 1990; Szeimies *et al.*, 1996). Although ALA-PDT has been successfully used for the treatment of superficial basal cell carcinoma and Bowen's disease, initial reports of PDT for SCC and nodular BCC have demonstrated relatively high rates of incomplete responses and a high rate of recurrence (McCaughan *et al.*, 1989; Pennington *et al.*, 1988).

For the treatment of cancers, ALA-PDT has been associated with minimal systemic toxicity (Gomer *et al.*, 1986). Photoactivation following ALA-PDT occurs only at sites exposed to light. This phenomenon limits potential adverse reaction occurring at other sites of PpIX accumulation after systemic administration of ALA. Moreover, the drug activating light (635 nm), which is in the range of visible light, is not known to induce adverse effects in the absence of the ALA-derived porphyrin after topical application of ALA (Kessel, 1996). In addition, since ALA-derived PpIX is present in the cytoplasm and does not accumulate in cell nuclei, ALA-based PDT has theoretically a low potential of causing DNA damage, mutations, and carcinogenesis (Moan, 1986).

### 1.2.1 History

Naturally occurring psoralen derivatives were used in combination with sunlight to treat various medical conditions in ancient Egypt, India, and China (Daniell and Hill, 1991). However, the first studies using PDT for the treatment of skin tumors were performed by German scientists in the early 1900's (Daniell and Hill, 1991). Meyer-Betz was the first to demonstrate long term clinical photosensitivity after injecting himself with hematoporphyrin in 1913 (Lui and Anderson, 1993). In 1942, Auler and Banzer used hematoporphyrin for the selective photodynamic destruction of tumors in animal models (Daniell and Hill, 1991). During the 1960s, Lipson studied the tumor localizing properties of hematoporphyrin derivative (HPD) (Lui and Anderson, 1993).

Contemporary interest in PDT developed in the 1970's with the first description of long term, PDT-induced tumor cure by Dougherty and collaborators (Dougherty *et al.*,

1975). Dougherty demonstrated that hematoporphyrin derivative (HPD) and red light could selectively destroy tumor cells without destroying normal tissue (Dougherty, *et al.*, 1975). Malik and Lugaci pioneered aminolevulinic acid (ALA)-based photosensitization of cells *in vitro* in 1987 (Malik and Lugaci, 1987). In 1990, Kennedy *et al.*, first applied topically ALA-based PDT clinically (Kennedy, *et al.*, 1990). Since then, there has been an intense research interest in ALA-PDT which culminated this year in the approval of this therapy for the treatment of actinic keratoses by the US Food and Drug administration (Kennedy *et al.*, 1996; Peng *et al.*, 1997).

### 1.2.2 Photosensitizers

A large number of photosensitizing agents have been tested *in vitro* and *in vivo* in PDT experiments. Ideally, photosensitizers should possess the following properties: (1) selective uptake and retention in diseased tissue; (2) rapid clearance from normal tissue, particularly the skin and eyes; (3) activation at longer, more penetrating wavelengths of light; (4) no dark toxicity, and (5) low cost.

Hematoporphyrin derivative (HPD) and porfimer sodium belong to the first generation of PDT photosensitizers (Dougherty *et al.*, 1998). HPD was the first photosensitizer used to treat skin diseases (Dougherty and Marcus, 1992). Porfimer sodium is one of the most widely studied photosensitizers and until recently was the only photosensitizer approved for clinical use. Porfimer sodium, causes prolonged cutaneous photosensivity which has limited its use in dermatology (Bissonnette and Lui, 1997). Porfimer sodium has been approved in several countries for the treatment of certain

pulmonary, gastrointestinal, and genitourinary malignancies (Dougherty, *et al.*, 1998). Other photosensitiezers which are currently being studied in man include Benzoporphyrin derivative monoacid ring A (BPD-MA) (Leung, 1994), tin ethyl etiopurpurin (SnET2) (Rifkin *et al.*, 1997), and phthalocyanines (Allemann *et al.*, 1997).

#### 1.2.2.1 Aminolevulinc Acid (ALA)

ALA is a non-photoactive endogenous molecule that is a precursor of protoporphyrin IX in the heme biosynthetic pathway. ALA is formed from glycine and succinyl-CoA by ALA synthetase (Figure 1.1) (Bissonnette and Lui, 1997). The last step of this pathway is the incorporation of iron into PpIX, which takes place in the mitochondria under the action of the enzyme ferrochelatase (Bissonnette and Lui, 1997). The addition of exogenous ALA bypasses the rate-limiting step and gives rise to intracellular accumulation of PpIX, a highly photoactive molecule (Marcus *et al.*, 1996). The high fluorescence yield of PpIX enables the visualization of PpIX in skin following ALA administration using simple hand-held UVA lamp (Wood's lamp) (Figure 1.2). PpIX fluorescence is seen as a pink-red color in a background of blue or violet light (Kennedy and Pottier, 1992).

Malignant tissues have been reported to exhibit a higher activity of porphobilibogen deaminase, a decreased ferrochelatase activity and a lower concentration of iron relative to normal tissues (Rittenhouse-Diakun, *et al.*, 1995; Van Hillegersberg, *et al.*, 1992). Therefore the higher rate of PpIX fluorescence in tumors may represent a higher capacity for conversion of ALA to PpIX or a decrease in transformation of PpIX

into heme. Abels *et al.* (Abels *et al.*, 1997) and Fritsch *et al.* (Fritsch *et al.*, 1997), have also suggested that selective PpIX accumulation in tumors is due to active uptake of ALA.

A preferential distribution of ALA-derived porphyrins in tumor cells provides the possibility of in situ detection of the porphyrin fluorescence in the superficial transformed epithelial cells of the skin and internal hollow organs by means of fibroptic point monitoring systems or of fluorescence imaging systems (Pottier *et al.*, 1986). The method of *in vivo* fluorescence spectroscopy is a rapid and useful technique that may be readily applied to obtain information related to the pharmacokinetic behavior of fluorescent drugs. It must be remembered, however, that surface detected fluorescence represents a summation of emission that may originate from several tissue and cell types.

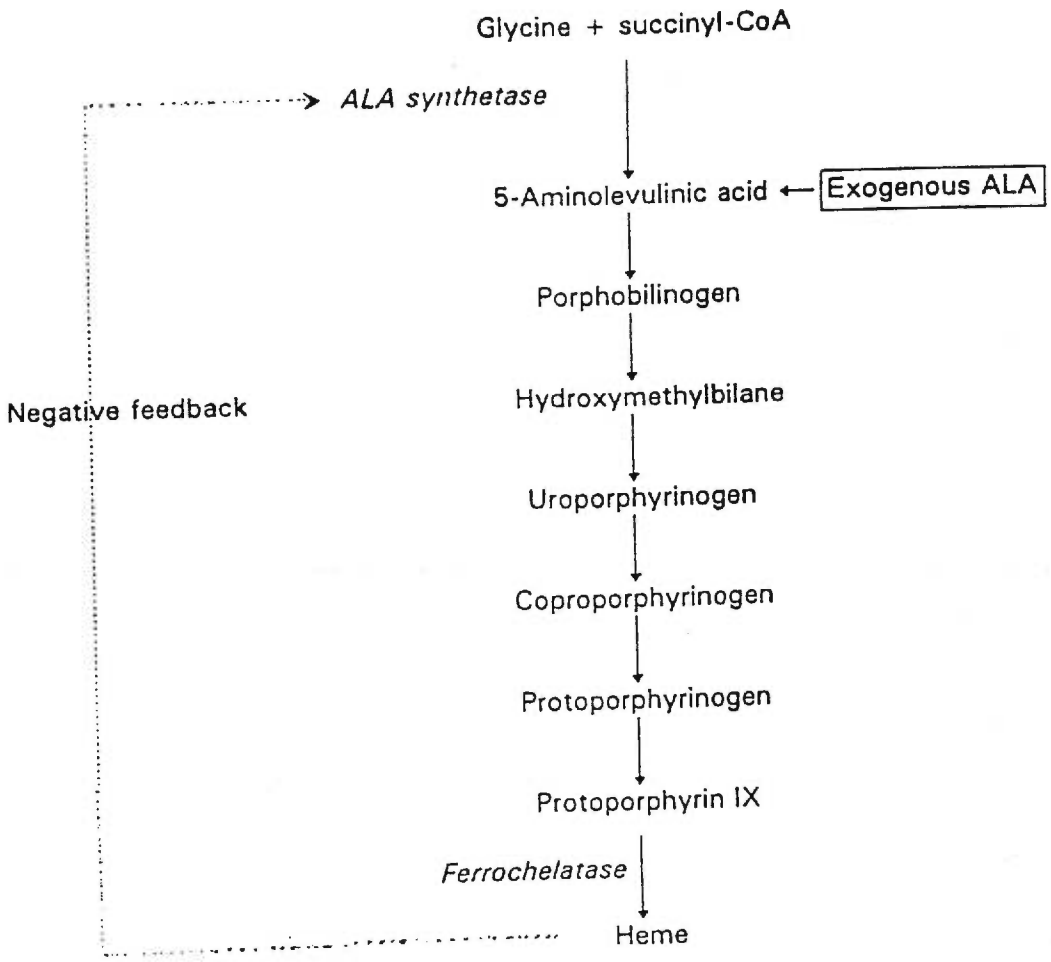


**Figure 1.1.** Heme biosynthetic pathway

(Bissonnette *et al*, Dermatologic Clinics, 1997; 15: 507-519)

**Figure 1.2:** Red fluorescnce with Wood's lamp on mouse skin (right side).

Topical application of ALA to cutaneous tumors gives rise to higher levels of PpIX accumulation in tumor as compared to adjacent skin (Kennedy, *et al.*, 1990). The main advantages of topical ALA-PDT are the absence of long-term systemic photosensitivity (Bissonnette and Lui, 1997) and the ability to complete treatment with both drug and light on the same day as an office procedure. However, low drug penetration through intact skin limits the potential of topical ALA-PDT (Peng, *et al.*, 1997). Heterogeneous protoporphyrin IX accumulation in tumors after topical ALA could perhaps explain the lack of efficacy in larger tumors (Martin *et al.*, 1995). This problem may be overcome by systemic administration of ALA (Peng, *et al.*, 1997).

Oral ALA has been used to treat benign and malignant oral and gastrointestinal tumors (Gossner *et al.*, 1995; Grant *et al.*, 1993; Mlkvy *et al.*, 1995; Regula *et al.*, 1995). When given to patients with BCC, systemic ALA generates a more homogenous distribution of protoporphyrin IX fluorescence than topical ALA (Peng *et al.*, 1995; Tope *et al.*, 1998) suggesting that higher cure rates may be achieved with systemic ALA.

### 1.2.3 Light source in PDT

To induce PDT effects, the spectral output of the light source must include wavelenghts that activates the photosensitizer. Both laser and nonlaser light sources can be used for PDT. A laser offers significant advantages whenever fiber optics are needed to reach the tumor. In dermatology, the most widely used laser systems are the argon ion pumped dye laser (argon-PDL) (630 nm) and the gold vapor laser (628 nm) (Fisher *et al.*, 1995). There are some limitations to laser sources for PDT in dermatology. Lasers are

expensive and somewhat complicated devices. Moreover, it can be difficult to treat large or multiple areas of skin with lasers because of their relatively low average output (Fritsch *et al.*, 1998). Therefore, in the treatment of large skin lesion, incoherent light devices are superior to laser systems (Fritsch, *et al.*, 1998). Incoherent sources used in PDT include arc lamps (e.g. xenon arc solar simulators) and incandescent lamps (e.g. slide projectors), which are inexpensive and readily available.

### 1.2.4 Mechanism of action

The efficacy of PDT is dependent on several factors including the absorption spectrum of the photosensitizer, the wavelength of activating light, the presence of competing chromophores, the depth of tissue penetration by light, and the biologic response of tissue. Each photosensitizer has a unique excitation spectrum, and the light source must be at the appropriate wavelength (s) to activate the photosensitizer in order to generate a PDT effect. In the case of ALA, PpIX fluorescence is usually excited with either blue (400-420 nm) or red light (620-640 nm) (Szeimies *et al.*, 1995). The depth of penetration increases with increasing wavelength. For most tissues, the photodynamic effect of red light at 630 nm is probably limited to the outer 1 cm of tissue (Frazier, 1996). By increasing the wavelength of the light source to 800 nm, the effective depth of penetration would be approximately doubled to 2 cm (Frazier, 1996).

### 1.2.4.1 Role of oxygen

Photodynamic interactions will take place wherever sensitizers, light of appropriate wavelength and oxygen are present simultaneously. Following the absorption of light, the photosensitizer is transformed from its ground state (singlet state) to an electronically excited state (triplet state). The exited triplet can then either react with a substrate to create free radicals (type 1 reaction) or transfer its energy to oxygen directly to form highly reactive oxygen species (type 2 reaction) (Henderson and Dougherty, 1992). Type 2 reactions appear to be the predominant mechanism in PDT (Henderson and Dougherty, 1992).

Reactive oxygen species can damage the vascular endothelium and cellular membranes through lipid peroxidation and protein damage (Nelson *et al.*, 1988). It has been estimated that singlet oxygen can only migrate about 0.02 μm after its formation, suggesting that cytosol located singlet oxygen following activation of PpIX can not induce direct damage to nuclear DNA (Dougherty, *et al.*, 1998).

The availability of molecular oxygen plays an essential and rate-limiting role in sustaining photodynamic intracellular processes (Gomer and Razum, 1984; Henderson and Dougherty, 1992). PDT effects are decreased or abolished at low oxygen concentrations (Henderson and Dougherty, 1992). *In situ* consumption of tissue oxygen during light exposure may itself be a rate-determining factor for PDT. This rapid decrease in oxygen tension during PDT is directly proportional to tissue photosensitizer level and light fluence rate (Tromberg *et al.*, 1990). Treatment protocols using low fluence rates or

intermittent light exposure have shown superior efficacy in cell culture and animal models (Blant *et al.*, 1996; van Geel *et al.*, 1996). At low light dose, repair of sublethal damage can occur, whereas at high doses, oxygen depletion can decrease therapeutic effect. Therefore, reduction of fluence rate or fractional irradiation may increase PDT efficacy because of increased singlet oxygen levels.

#### 1.2.4.2 Photobleaching

Following light activation the structure of photosensitizers can be modified in such way that the photosensitizing properties of the molecule are lost. This is termed photobleaching and represents one mechanism whereby the photodynamic reaction can be turned off. (Potter *et al.*, 1987). This phenomenon can be used to enhance PDT efficacy by photobleaching the lesser amount of photosensitizer in normal tissue, whereas the tumor is photodynamically destroyed because of higher photosensitizer level (Mang *et al.*, 1987).

#### 1.2.4.3 Role of vascular photodamage

For photosensitizers that mainly localize to the vascular compartment, direct damage to the endothelial cells with subsequent vascular shutdown plays a more important role in tumor destruction than direct cytotoxicity (Fingar *et al.*, 1992). This is the case of porfimer sodium (Fingar, 1996; Herman *et al.*, 1999; Roberts *et al.*, 1994; van Geel *et al.*, 1994). The vascular effect is not as important with systemic ALA-PDT (Bedwell *et al.*, 1992; Herman, *et al.*, 1999; Leveckis *et al.*, 1995; Roberts, *et al.*, 1994). However, damage to the endothelium and the basal lamina of vascular walls has been

observed by light and electron microscopy following ALA-mediated PDT in animal models (Peng *et al.*, 1992). Roberts *et al.* (Roberts, *et al.*, 1994) have also reported the presence of porphyrin in or near the vascular wall in animal models following ALA application. In patients, it has been shown the reduction of tumor blood flow after ALA-PDT (Herman, *et al.*, 1999).

#### 1.2.4.4 Apoptosis

Apoptosis is a genetically programmed cell death resulting in nuclear DNA fragmentation, chromatin condensation and cell involution with minimal or no associated inflammation (Cohen, 1993). Apoptosis has been the subject of intensive investigations during the past several years. Apoptosis has been shown to occur in cell culture after PDT with aminolevulinic acid (Noodt *et al.*, 1996), porfimer sodium (He *et al.*, 1994), and aluminium phthalocyanine (Agarwal *et al.*, 1991). PDT-induced cytotoxic damage appears to be dependent on the subcellular localization of each photosensitizer. Photosensitizers that preferentially localize to the mitochondria, such as porfimer sodium and aminolevulinic acid (ALA), are more likely to induce apoptosis while sensitizers localizing to the plasma membrane are likely to cause necrosis following light exposure (Kessel and Luo, 1999; Kessel *et al.*, 1997).

### 1.2.4.5 Inflammatory response

PDT-induced inflammation and anti-tumor immune response are also thought to play key roles in PDT response (Lui and Anderson, 1993). The mild to moderate erythema and swelling that develops at the treated sites less than 24 hours after PDT is a clinical sign of inflammation (Lui and Anderson, 1993). PDT with porfimer sodium has been shown to exert a direct cytotoxic effect on macrophages that stimulates tumor necrosis factor production (Evans *et al.*, 1990). PDT with porfimer sodium can induce production of interleukin 2 (IL-2), IL-1β, IL-6, and IL-10 and TNF-α in both *in vitro* and *in vivo* experiments (Dougherty and Marcus, 1992),(Gollnick *et al.*, 1997).

PDT-induced tumor-specific immune response may not be relevant to the initial tumor ablation, but may be decisive in attaining long-term tumor control (Korbelik, 1996). This effect may contribute to the elimination of small tumor foci left viable by PDT direct cytotoxic effect (Korbelik, 1996).

### 1.2.5 Adverse effects

Photosensitivity is the major adverse effect encountered after systemic administration of a photosensitizer. The duration of photosensitivity varies among photosensitizers with an average of 4-6 weeks for porfimer sodium (Dougherty and Marcus, 1992) 2-3 days for BPD (Marcus *et al.*, 1994), and less than 48 hours for systemic ALA (Marcus, *et al.*, 1996). Following exposure to sunlight, reactions vary from mild burning or stinging to severe edema, erythema, and bulla formation. While localized skin necrosis is acceptable for cancer treatment, generalized necrosis is

unacceptable for whole body PDT such as for the treatment of psoriasis or for skin cancer prevention. Scarring, hyperpigmentation and hypopigmentation at treatment sites are also possible (Wilson *et al.*, 1992). Pain at the treatment can occur with high drug-light combinations but is of short duration and can usually be well controlled with mild analgesics (Wilson, *et al.*, 1992).

Much less is known about side effects associated with systemic ALA administration. It appears that oral administration of ALA (< 60 mg/kg) or intravenous infusion (< 30 mg/kg) does not lead to any neurotoxic symptoms, although some patients may have mild, transient nausea and/or temporary abnormalities of liver functions (Peng, *et al.*, 1997).

### 1.3 Rationale and Objectives

As ALA induces the synthesis of PpIX preferentially in cancer cells, it could theoritically be effective in the treatment of skin cancer at the microscopic stage, before it becomes clinically visible. Indeed, treatment of Aks (precursors of SCC) by ALA-PDT could be constructed as a form of chemoprevention to the extent that treating AKs may prevent transformation to SCC. Because most skin cancers are present in sun exposed regions, the administration of ALA followed by its activation by a sub-erythematous light exposure could trigger a low level, chronic photodynamic therapy that could induce destruction of microscopic cancer cells, while being insufficient to provoke erythema on normal skin. One major advantage of ALA-PDT is that PpIX can be activated by visible light, suggesting that patients at higher risk of skin cancer could eventually be given this

drug and achieve a PDT effect from visible light without having to expose themselves to solar irradiation containing carcinogenic UV irradiation.

The previously mentioned clinical studies demonstrating that ALA can treat AKs, Bowen's, SCC, support the use of our chosen mouse model where SCC is the predominant UV-induced skin tumor.

In order to find experimental conditions where weekly whole body PDT could delay the appearance of skin cancer without increasing the mortality or the incidence of large tumors we have studied the ability of weekly sub-erythematous PDT with systemic ALA to prevent the appearance of UV induced skin cancer in hairless mice.

## Chapter II

### Systemic photodynamic therapy with aminolevulinic acid delays the appearance of UV induced skin tumors in mice.

## 2.1 Abstract

Photodynamic therapy (PDT) with topical aminolevulinic acid (ALA) has recently been approved by the US Food and Drug Administration for the treatment of actinic keratoses. The purpose of this study was to determine whether weekly systemic sub-erythemogenic ALA-PDT could prevent the appearance of UV-induced skin tumors in hairless mice. Groups of 20 mice were administered daily UV radiation from FS20 tubes, and weekly intraperitoneal injections of ALA at 40 mg/kg, each followed 3 hours later by 12 J/cm$^2$ of white light (ALA-PDT). The tumor free survival was significantly longer for mice exposed to daily UV and weekly ALA-PDT as compared to mice exposed only to UV, to UV and ALA without white light, or UV and white light without ALA. Neither the mortality nor the incidence of large skin tumors were higher in the UV-ALA-PDT group. *In vivo* fluorescence spectroscopy showed that the 635 nm fluorescence emission within tumors was lower than in normal skin 3 hours after ALA administration. This was also confirmed by quantitative fluorescence microscopy, suggesting that PDT performed at 40 or 60 minutes after ALA administration, when the tumor : normal skin fluorescence ratio is higher, could further delay the appearance of UV-induced skin tumors.

## 2.2 Introduction

Skin cancer is the most common human cancer (Gloster and Brodland, 1996). The current approach for skin cancer prevention is mainly focused on sun avoidance, sun protection with sunscreens and clothing, as well as frequent medical visits for early tumor detection. There is a need for additional preventive strategies for patients at high risk for developing skin cancer, including patients with a previous history of multiple skin cancers, post-organ transplant immunosuppression, or genetic disorders such as nevoid basal cell carcinoma syndrome or xeroderma pigmentosum.

Photodynamic therapy (PDT) is a recently approved therapeutic modality combining the administration of a photosensitizer with its subsequent activation by light to generate highly reactive oxygen intermediates (Bissonnette and Lui, 1997). PDT with aminolevulinic acid (ALA), a precursor of the photosensitizer protoporphyrin IX (PpIX), has been recently approved by the US Food and Drug Administration for the treatment of actinic keratoses. Complete responses have also been reported with ALA-PDT for the treatment of superficial basal cell carcinoma and Bowen's disease (Cairnduff *et al.*, 1994; Calzavara-Pinton, 1995; Fijan *et al.*, 1995; Jeffes, *et al.*, 1997; Kennedy, *et al.*, 1990; Svanberg *et al.*, 1994; Wolf *et al.*, 1993). Repeated erythemogenic PDT sessions with topical ALA has recently been shown to delay the appearance of UV-induced skin cancer in mice (Stender *et al.*, 1997). However an increased in mortality and a higher incidence of large tumors were observed for ALA-PDT treated mice in this study. We hypothesize that weekly sub-erythemogenic PDT with systemic ALA could destroy microscopic skin cancers before they evolve into clinically apparent tumors, thereby preventing the

development of visible skin cancer without increasing mortality or the incidence of large tumors.

## 2.3 Materials and Methods

### Chemicals and Reagents

5-Aminolevulinic acid (5-ALA) was purchased from Sigma (St. Louis, MO) and dissolved in normal saline immediately prior to intraperitoneal injection.

### Animals

Eight weeks old female SKH1 hairless mice were purchased from Charles River Laboratories (St-Contant, Canada). All experiments were approved by the University of Montreal Hospital Center Animal Care Committee. All mice were fed a standard rodent diet and kept on a 12 hour on/off ambient light cycle unless injected with ALA, in which case the mice were kept in the dark for 24 hours after the injection.

### Ultraviolet exposure and photodynamic therapy

The animals were divided into eight groups of 20 mice each (Table 2.1). Groups A, B, C, and D were exposed six days per week to 163 $mJ/cm^2$ of UV radiation from a bank of four FS 20 sun lamp tubes (Westinghouse, Bloomfield, NJ). This fluence corresponds to 90% of the minimal erythemal dose (data not shown). The irradiance was measured weekly with an International Light IL 1700 radiometer (Newburyport, MA) equipped with a SED 240 detector, a UVB-1 filter and a "Wide Eye" Diffuser (W). Mice from groups A, B, E, and F received a weekly intraperitoneal injection of ALA at 40 mg/kg.

Three hours after ALA injection, mice from groups A and E were exposed weekly to 12 J/cm$^2$ of broadband white light generated by a slide projector (Kodak Carousel 650 H, Toronto, ON, Canada). The irradiance was measured with the International Light IL 1700 radiometer equipped with a SED 033 detector, a F/CB248 filter and a "Wide Eye" Diffuser (W). UV exposures and weekly PDT were stopped if the tumor load was greater than 1 cm$^3$, if large eroded tumors were present, if erosions developed on the backs of the mice, or after 24 weeks of UV exposure, whichever came first.

**Tumor recording and analysis**

Mice were examined weekly for the presence of skin tumors. All tumors were recorded by location and size. Any tumor of more than 1 mm diameter that was present for at least 2 consecutive weeks was included in the analysis. Tumor incidence defined as the percentage of tumor-bearing mice per group was used for statistical analysis.

**Histopathology**

Representative specimens of small (1mm), medium (2-4 mm), large (≥ 4 mm) tumors, and UV-exposed skin were excised, fixed in 10% buffered formalin, processed for routine histopathology. Sections were stained with hematoxylin and eosin.

**In vivo fluorescence spectroscopy**

Twenty tumor-bearing mice from groups A, B, C, or D were injected intraperitoneally with 40 mg/kg of freshly made ALA. A Skin Skan spectrofluorometer (SPEX model, Instruments SA, Edison, NJ) equipped with a Xenon lamp and 2

monochromators was used for *in vivo* fluorescence spectroscopy. Skin fluorescence was excited at 410 nm and the intensity of the emission at 635 nm for PpIX was recorded for baseline and at 10 min, 20 min, 40 min, 1 h, 1.5h, 2h, 3h, 4h, 6h, 8h, 10h, and 24 hours after ALA injection. At each time point, fluorescence intensity was measured at 3 different sites per mouse for UV-exposed skin, and for small, medium, and large tumors. The same measurements were also performed on the backs of five mice with no UV exposure and no tumors. All animals were kept in the dark throughout this experiment.

**Fluorescence microscopy**

Twelve mice from groups D (UV) and H (control) received a 40 mg/kg intraperitoneal injection of ALA and were sacrificed in groups of 3 at the following times after injection: 40 minutes, 1 hour, 2 hours and 3 hours. Four specimens of UV-exposed back skin, small tumors, and normal unexposed back skin (for mice in group H) were embedded in Frozen Section Medium (Stephens Scientific, Riverdale, NJ) and frozen in liquid nitrogen. Five sections of 10 μm thickness from each frozen sample were cut with a cryostat and stored at – 70° C. Tissue sections were prepared with a minimum of light exposure in order to avoid photobleaching of PpIX. Fluorescence images were generated with a Nikon Optiphot-2 fluorescence microscope equipped with a thermoelectrically cooled CCD camera (Model DC330E, Dage-MTI Inc, Michigan city, IN) hooked up to a PC computer. The integration time was 1 second, which did not cause significant photobleaching (not shown). For fluorescence recording, a 480 nm short pass (480DF60) excitation filter and a 635±10 nm band pass (635DF55) emission filter were used (Harvard Apparatus, Montreal, QC, Canada). After fluorescence recording, the slide was

stained with toluidine blue and a standard light microscopic image of the same area was recorded without moving the microscope stage. All images were analyzed with Clemex vision software (Version 3.0.023, Longueuil, QC, Canada). Using this software, the epidermis or tumoral areas were delineated on the toluidine blue images, transferred onto the fluorescence images, and the fluorescence intensity per unit area then measured.

**Statistical analysis**

The probability of tumor free survival was calculated using the Kaplan-Meier survival curve according to the number of weeks of treatment. The log rank test with Bonferroni correction was used to compare survival curves between the treatment groups. The ANOVA with Bonferroni correction was used to compare the number of tumor per mouse at 22 weeks after UV exposure. The one way ANOVA for repeated measures was used to analyze the results for *in vivo* fluorescence spectroscopy and fluorescence microscopy.

## 2.4 Results

### 2.4.1 Systemic ALA-PDT delays the appearance of UV-induced skin tumors

Histopathologically *in situ* squamous cell carcinoma with numerous atypical keratinocytes and mitoses with minimal or no dermal invasion were present in UV-exposed skin as well as within small (1-2 mm) tumors. Tumors greater than 2 mm demonstrated invasive squamous cell carcinoma (SCC) with proliferation of atypical neoplastic cells, numerous mitoses and a variable degree of differentiation. Tumors were not observed in mice that received only ALA, only ALA-PDT, only white light, or no treatment. The first tumors were observed in groups B and D after 8 weeks and in group

C after 9 weeks of UV exposure. The first tumors in the ALA-PDT treated groups were induced at 11 weeks. There was a significant difference in tumor-free survival for mice exposed to UV and ALA-PDT as compared to mice exposed only to UV, to UV and ALA without white light, or UV and white light without ALA ($P = 0.0001$ with log rank test) (Figure 2.1).

Numerous small (< 1 mm) coalescent tumors were observed in all mice from UV-exposed groups except in the ALA-PDT group where only five mice showed coalescent small tumors. In addition, the number of tumors per mouse was significantly less at 22 weeks in group A (UV/ALA-PDT) than in other groups ($P < 0.05$) (Figure 2.2). Although this protocol was designed to administer sub-erythemal PDT and UV doses, erythema and/or erosions developed after 12 weeks in 10 mice in the UV /ALA-PDT group and in 2 mice from each of the UV/ALA, UV/white light, and UV only groups.

Six mice from the UV-ALA-PDT group were sacrificed because of skin erosions whereas all mice from the UV/ALA, UV/white light or UV only groups were sacrificed because of high tumor load or because of large (≥ 4 mm) eroded tumors. Only one mouse in the UV-ALA-PDT group presented a large tumor (≥ 4 mm) as compared to 7, 8, and 10 mice in groups B, C, and D respectively.

### 2.4.2 635 nm fluorescence kinetics is different for skin tumor versus normal and non-tumoral UV-exposed skin

Twenty minutes after intraperitoneal administration of 40 mg/kg of ALA, a 635 nm fluorescence emission peak could be detected with *in vivo* fluorescence spectroscopy in skin tumors. In normal skin this peak could not be detected until 40 minutes. The intensity of the fluorescence in tumors at 635 nm increased to a maximum at 90 min and then decreased to baseline levels at 8 hours (Figure 2.3). The 635 nm fluorescence intensity increased more slowly on normal skin as compared to tumoral skin and UV exposed skin, and was also almost back to baseline at 8 hours. The 635 nm fluorescence intensity of tumors was higher than UV-exposed skin and normal skin from the time of detection in tumors until 2 h after ALA administration. After 3 hours, the 635 nm fluorescence intensity of normal and UV-exposed skin exceeded the fluorescence of tumors. Fluorescence intensity was significantly higher in tumor than normal skin 40 min and 60 min after ALA administration ($P < 0.05$). The tumor : normal skin fluorescence ratio was $2.2 \pm 0.2$ and maximum 40 minutes after ALA injection and decreased to reach $0.63 \pm 0.1$ at 3 hours after ALA administration. The tumor : UV-exposed skin ratio was $1.48 \pm 0.26$ after 40 minutes of ALA injection and decreased to $0.78 \pm 0.1$ after 3 hours (Figure 2.4). Before sacrifice, the intensity of 635 nm fluorescence as well as the time of peak fluorescence in tumors, UV-exposed, and normal skin were compared for mice from groups A, B, C, and D. There was no statistical difference between the groups (data not shown).

### 2.4.3 Microscopic red fluorescence is lower in tumors as compared to normal and UV exposed epidermis 3 hours after ALA administration

Forty minutes after ALA administration, red fluorescence was observed on microscopy at the level of sebaceous glands, normal epidermis, UV-exposed skin, and tumors (Figure 2.5). Forty and 60 minutes after ALA injection, red fluorescence was higher in small tumors as compared to UV-exposed skin and normal skin ($P < 0.05$), whereas at 2 and 3 hours after ALA administration, red fluorescence was lower in tumors as compared to adjacent UV-exposed skin and normal skin ($P < 0.05$) (Figure 2.6). Red fluorescence at 2 and 3 hours after ALA injection was 2 and 3.5 times more intense on normal back skin, and 2 and 3 times more intense on UV-exposed skin respectively as compared to small tumors.

## 2.5 Discussion

Daily exposure of hairless mice to ultraviolet radiation induces multiple squamous cell carcinoma within about 3 months (de Gruijl and Forbes, 1995). Because of this short interval and their limited amount of hair and small size, hairless mice were selected as a model to study the effect of systemic ALA-PDT on the development of UV induced skin cancer. Although the hairless mouse is a practical model, there are differences between photocarcinogenesis in mice and humans, including the high daily UVB fluence used in this study and the longer time needed for the development of squamous cell carcinoma in humans as compared to mice.

Daily exposure of hairless mice to UV radiation alone induced tumors in all mice by week 22 in this study. The first tumors, 1 mm in diameter were mostly actinic keratoses whereas larger tumors were mostly squamous cell carcinomas as described previously (de Gruijl and van der Leun, 1991). Using weekly systemic ALA-PDT we were able to delay the appearance of UV induced skin cancer in the hairless mouse. This delay could not be attributed to either light exposure alone or weekly ALA injections without light exposure, as the tumor free survival curves of these groups paralleled those of mice exposed only to UV. Stender and colleagues reported a delay in the appearance of UV induced skin cancer using weekly PDT with topical application of 20% ALA in a cream base (Stender, *et al.*, 1997). However they observed a higher number of large tumors and an increased mortality for mice treated with topical ALA-PDT, findings which were not observed in this study. The higher incidence of large tumors reported by Stender *et al.*, could be related to inadequate penetration of topical ALA. In patients with basal cell carcinoma, systemic ALA at 40 mg/kg has been shown to induce homogenous tumor fluorescence as compared to heterogeneous and incomplete fluorescence after topical ALA application (Martin, *et al.*, 1995; Tope, *et al.*, 1998), and it is possible that topical ALA photosensitizes malignant tumors incompletely in hairless mice thereby allowing some tumor cells to grow despite light exposure. Our protocol was designed for sub-erythemogenic and sub-clinical PDT, as opposed to the protocol of Stender *et al.* where mice were believed to experience pain during PDT. During the last weeks of our experiments some mice in Groups A (UV-ALA-PDT), D (UV only), C (UV-light) and E (ALA-PDT) developed erythema or erosions on their back, suggesting that chronic UV-exposure as well as repeated ALA-PDT sessions may decrease the erythemal threshold

for UV and ALA-PDT respectively. A direct comparison between the tumor-free survival curves of Stender *et al.* and those of this study is difficult as the light source and the irradiation protocols were different.

*In vivo* fluorescence spectroscopy of normal and tumoral mouse skin performed after ALA injection showed an emission spectrum typical of PpIX with a peak at 635 nm. In order to determine the time course of tumor : normal skin PpIX fluorescence, this technique was applied to mice with UV-induced tumors. The back skin of non-UV-exposed mice was used for normal skin. Comparison to non-UV exposed skin may be an appropriate model for patients with skin cancer as histological analysis of non-tumoral skin in our mice exposed to UV for 22 weeks revealed confluent *in situ* squamous cell carcinoma whereas non-tumoral skin of patients with skin cancer usually shows at most a limited number of atypical keratinocytes. Although ventral skin of our experimental mice was not directly exposed to UV, this area was not used to determine the tumor : normal skin ratio as PpIX intensity was consistently lower over ventral as compared to dorsal skin of normal mice (not shown). *In vivo* fluorescence spectroscopy showed that at three hours after injection of ALA, the time of light exposure used in this study, PpIX fluorescence was lower in tumor than in adjacent UV-exposed skin and normal back skin. Maximal PpIX fluorescence occurred at 90 min in tumors and 3 hours in normal back skin. Maximum PpIX fluorescence intensity in rodent tumors after ALA injection varies between 1-6 h depending on the tumor model and the ALA dose administered (Abels *et al.*, 1994; Bedwell, *et al.*, 1992; Henderson *et al.*, 1995; Hua *et al.*, 1995; Peng, *et al.*, 1992). PpIX fluorescence intensity for mice with UV-induced tumors injected with 200

mg/kg of ALA has also been reported to peak earlier in tumors (2 hours for tumors and 5 hours for normal skin) (van der Veen *et al.*, 1994). In order to photoactivate PpIX preferentially in tumors, as opposed to adjacent skin, selection of an earlier time for light exposure after ALA injection could be desirable and may result in further delay in the appearance of UV induced tumors as well as further minimizing PDT phototoxicity.

Although *in vivo* fluorescence spectroscopy is a non-invasive and rapid technique for estimating PpIX levels in tumors, the recorded values could be influenced by the presence of PpIX fluorescence arising from non-epidermal sources such as sebaceous glands. Divaris *et al.* reported an elevated level of PpIX in sebaceous glands and hair follicles of mice after ALA injection (Divaris *et al.*, 1990) and we observed the same phenomenon in hairless mice (data not shown). In order to study if the lower tumor: normal skin PpIX fluorescence ratio observed at 3 hours was caused by a lower sebaceous contribution to total skin fluorescence within tumor-bearing sites (i. e., there is no sebaceous tissue in tumors themselves), quantitative fluorescence microscopy was performed. This analysis confirmed the lower tumoral : normal skin PpIX ratio at 3 hours thus excluding the possibilities that this observation was solely related PpIX fluorescence from sebaceous glands or hair follicles. According to microscopic fluorescence ratios measured at 40 min, 60 min and 2 hours, the best time to expose mice to light following ALA injection might be 40 or 60 min as the tumor : normal skin fluorescence ratio is highest at that time.

The mechanism by which ALA-PDT delays the onset of UV- induced skin cancer is unknown. After addition of ALA *in vitro*, PpIX accumulates preferentially in transformed cells as compared to their normal counterparts, perhaps in relation to lower iron concentrations and lower ferrochelatase activities in neoplastic cells (Rittenhouse-Diakun *et al,* 1995; Van Hillegersberg *et al,* 1992). PDT with ALA induces apoptosis in neoplastic cultured cells (Noodt, *et al.*, 1996) and it is possible that weekly systemic ALA-PDT delays the appearance of UV induced skin cancer by inducing apoptosis in malignant keratinocytes. A vascular effect is also possible as PpIX is present in the blood after systemic ALA administration (Rick *et al.*, 1997; Webber *et al.*, 1997) and a reduction in blood flow has recently been described in human colonic adenocarcinoma after systemic ALA-PDT (Herman, *et al.*, 1999). PDT with porfimer sodium can induce cytokine release in mice including tumor necrosis factor alpha, IL-6 and IL-10 (Gollnick, *et al.*, 1997) and the contribution of these cytokines to single and multiple ALA-PDT sessions deserves further study.

The low tumor : normal skin PpIX ratio at the time of light exposure could explain the absence of complete tumor prevention of in this study. Limited penetration of ALA in some tumors, the presence of PDT-resistant cells induced by chronic UV exposure as well as the selection of PDT resistant clones by multiple ALA-PDT sessions are all possible explanations for the absence of complete prevention that merit further study.

In conclusion this study showed that systemic ALA-PDT could delay the appearance of UV-induced skin cancer in hairless mice. Further studies addressing the optimization and mechanism of action for this delay will need to be performed before this type of photochemoprevention with PDT could be explored in a clinical setting.

| Group | Daily UV exposure | Weekly ALA injection | Weekly light exposure |
|---|---|---|---|
| A | Yes | Yes | Yes |
| B | Yes | Yes | No |
| C | Yes | No | Yes |
| D | Yes | No | No |
| E | No | Yes | Yes |
| F | No | Yes | No |
| G | No | No | Yes |
| H | No | No | No |

**Table: 2.1**

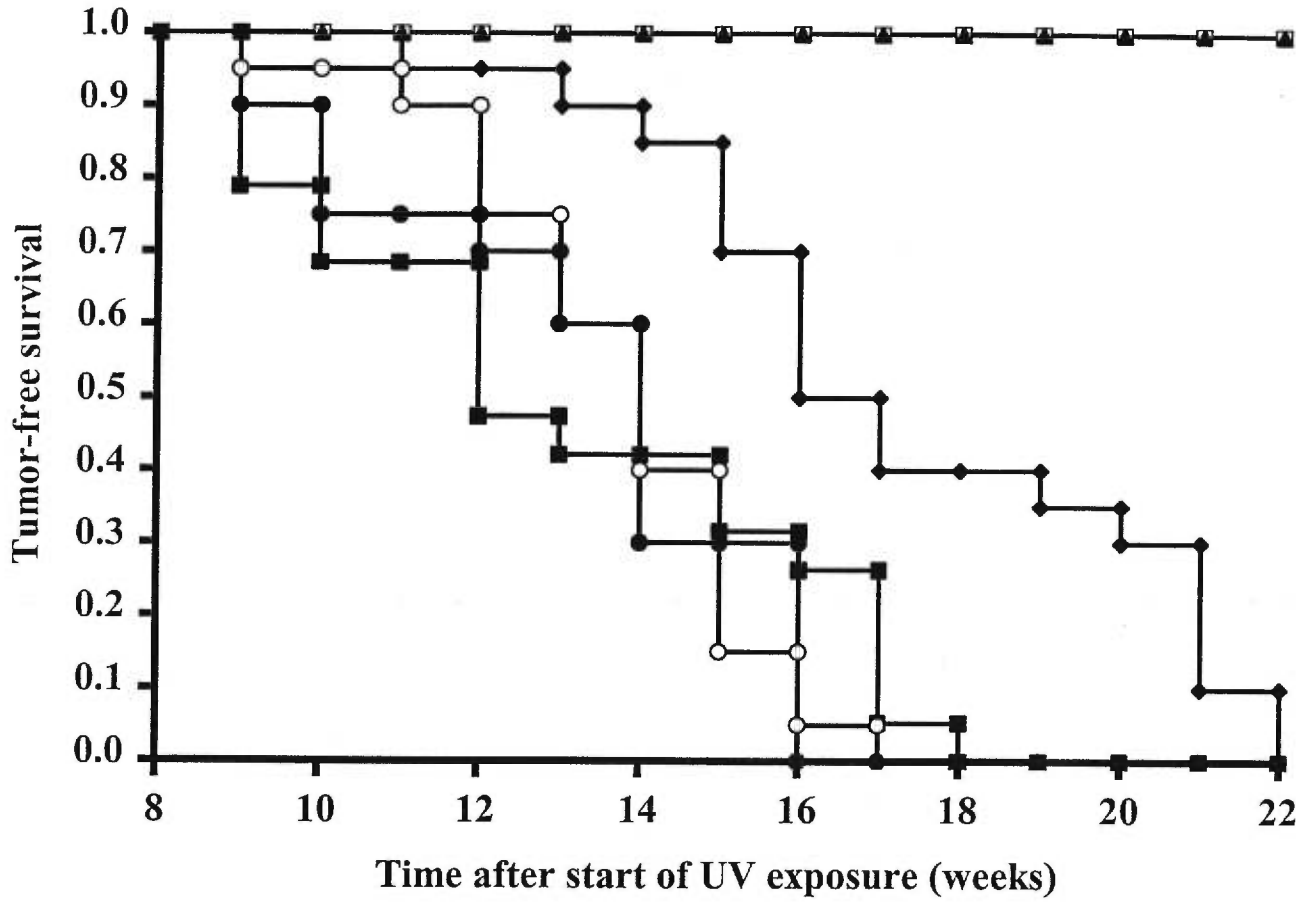


**Figure 2.1. Weekly photodynamic therapy with systemic ALA delays the appearance of UV-induced tumors**. Tumor-free survival according to the number of weeks after the start of UV exposure for UV/ALA-PDT (◆), UV/ALA (■), UV/Light (○), UV (●), ALA-PDT (□),Control (▲).

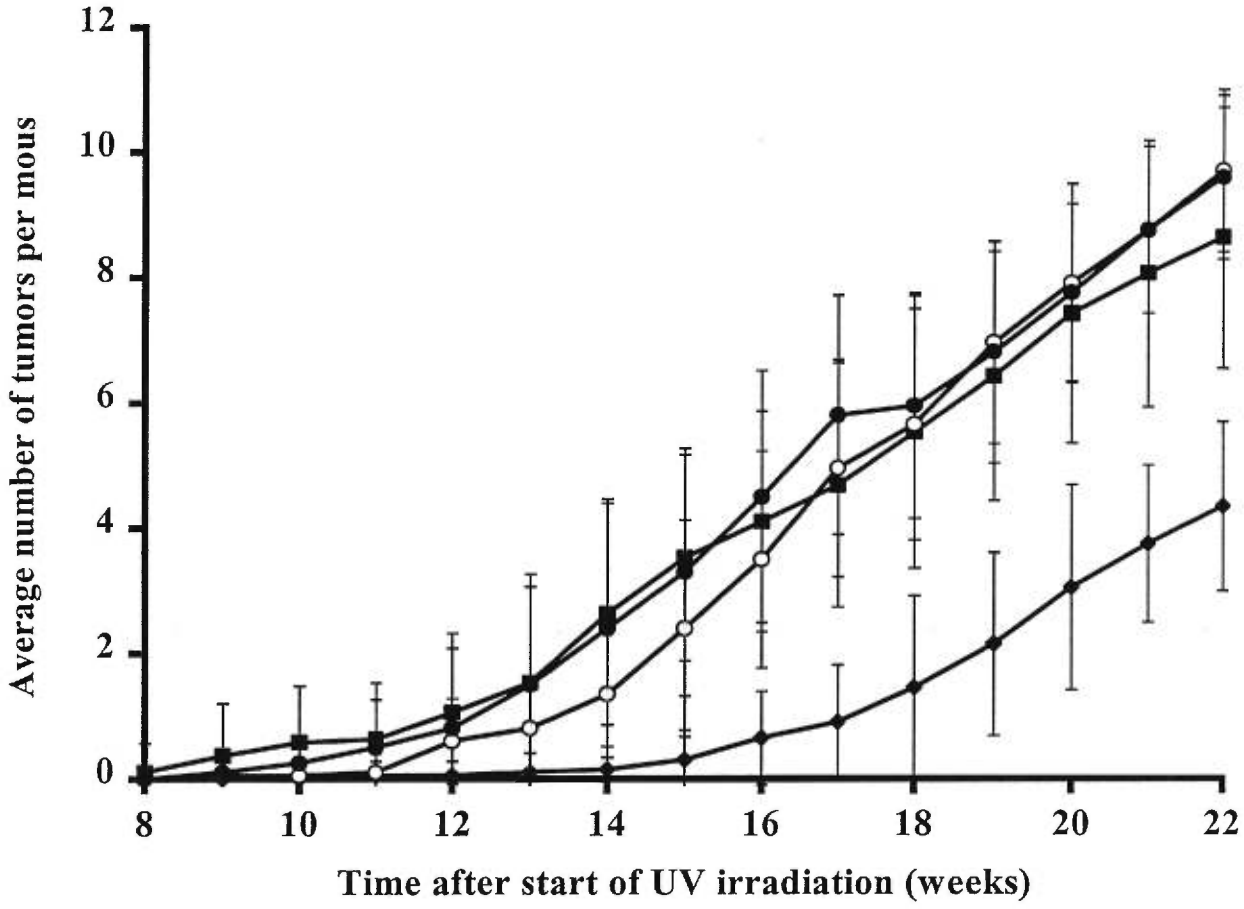


**Figure 2.2. The average number of tumor per mouse is lower after 22 weeks of UVB in mice treated weekly with systemic ALA-PDT.** Number of tumor per mouse according to time after the start of UV exposure for UV/ALA-PDT (◆), UV/ALA (■), UV/Light (O), UV (●).The data represent mean values (± SD) from 20 mice.

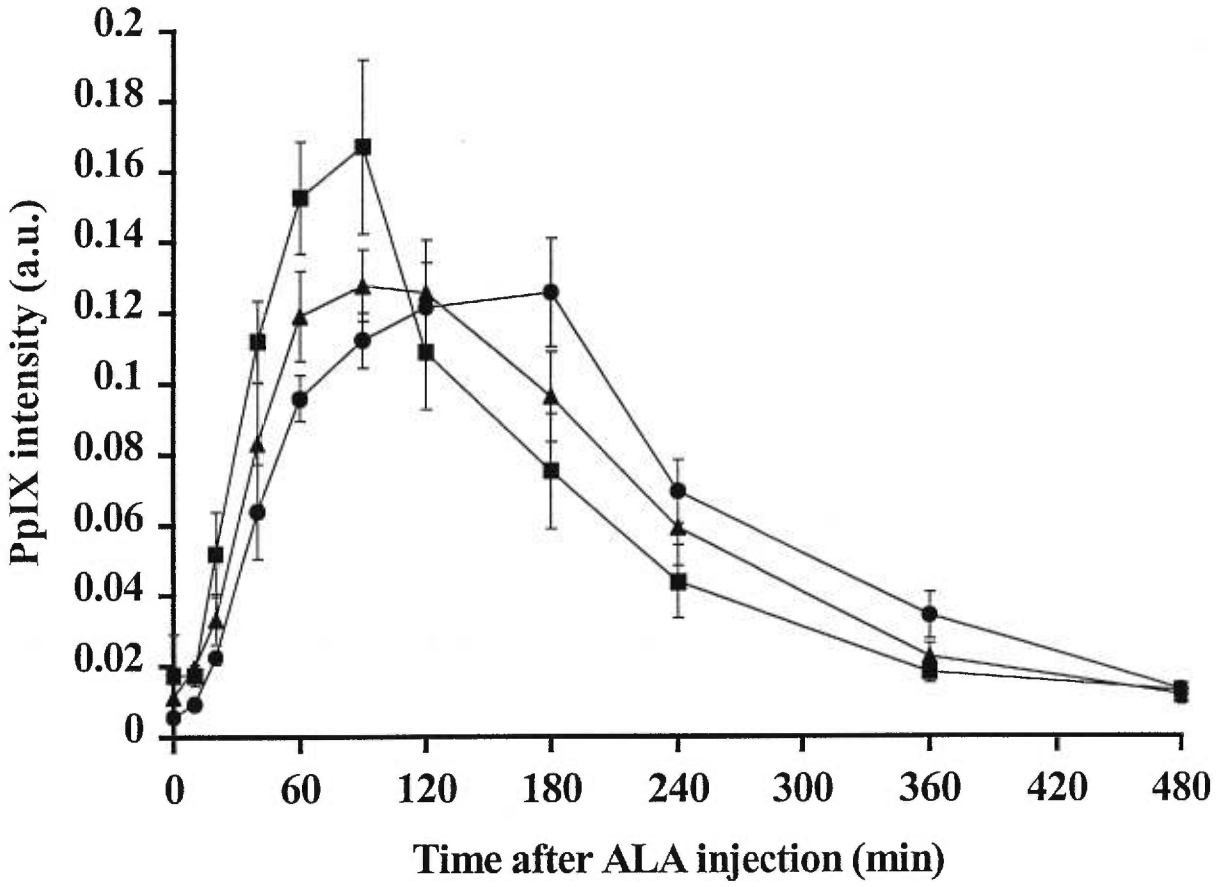


**Figure 2.3. Protoporphyrin IX fluorescence kinetics for normal skin, UV-exposed skin and skin tumors are different.** Intensity of PpIX fluorescence as measured by *in vivo* fluorescence spectroscopy after administration of 40 mg/kg ALA intra peritoneally for UV-exposed skin (▲), Small tumor (■), Normal back skin (●).The data represent mean values (± SEM) from three experiments for five mice.

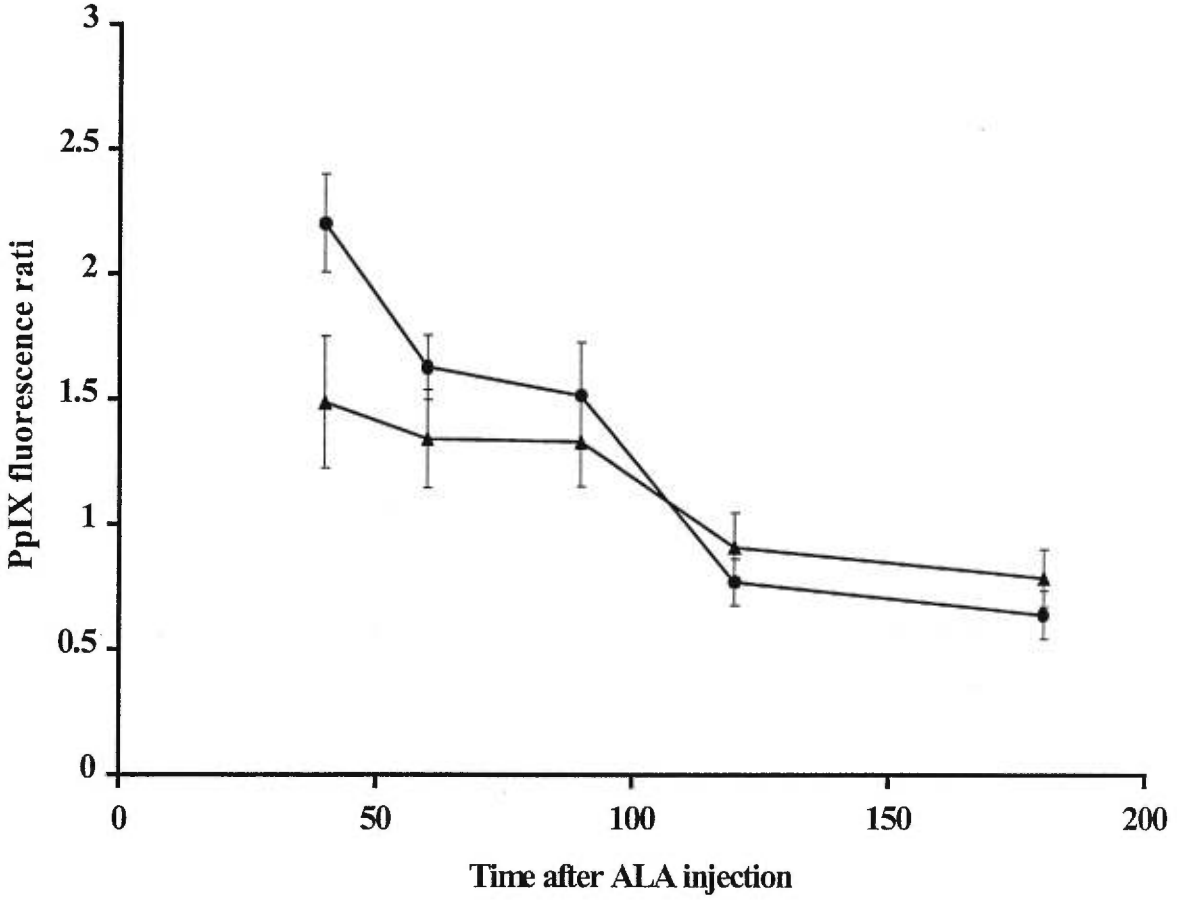


**Figure 2.4. Small tumor : normal skin protoporphyrin IX fluorescence ratio is highest at 40 minutes after IP injection of ALA at 40 mg/kg.** PpIX fluorescence ratio according to time after IP injection of ALA at 40 mg/kg as measured with *in vivo* fluorescence spectroscopy for Small tumor : normal skin fluorescence ratio (●), Small tumor : UV-exposed skin fluorescence ratio (▲). The data represent mean values (± SEM) from three experiments for five mice.

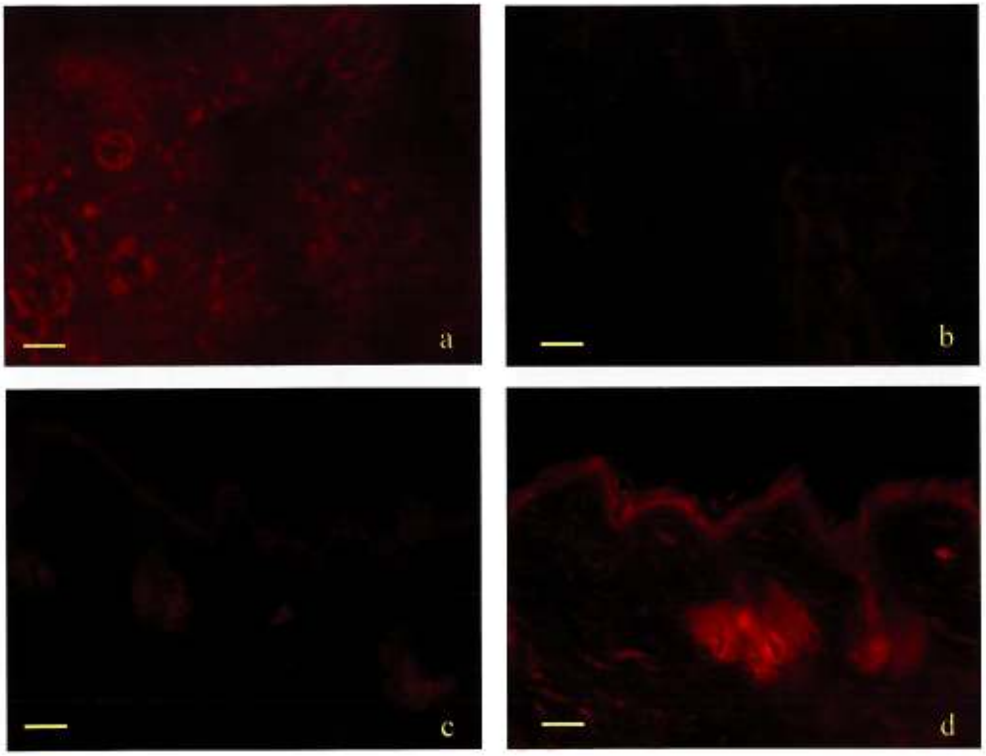


**Figure 2.5. Protoporphyrin IX fluorescence 3 hours after IP injection of ALA at 40 mg/kg is higher in normal skin than in small tumor.** Fluorescence microscopy of normal skin (c, d) and small tumor (a, b) at 40 minutes (a, c) and 3 hours (b, d) after IP ALA at 40 mg/kg. *Scale bar*: 50 μm.

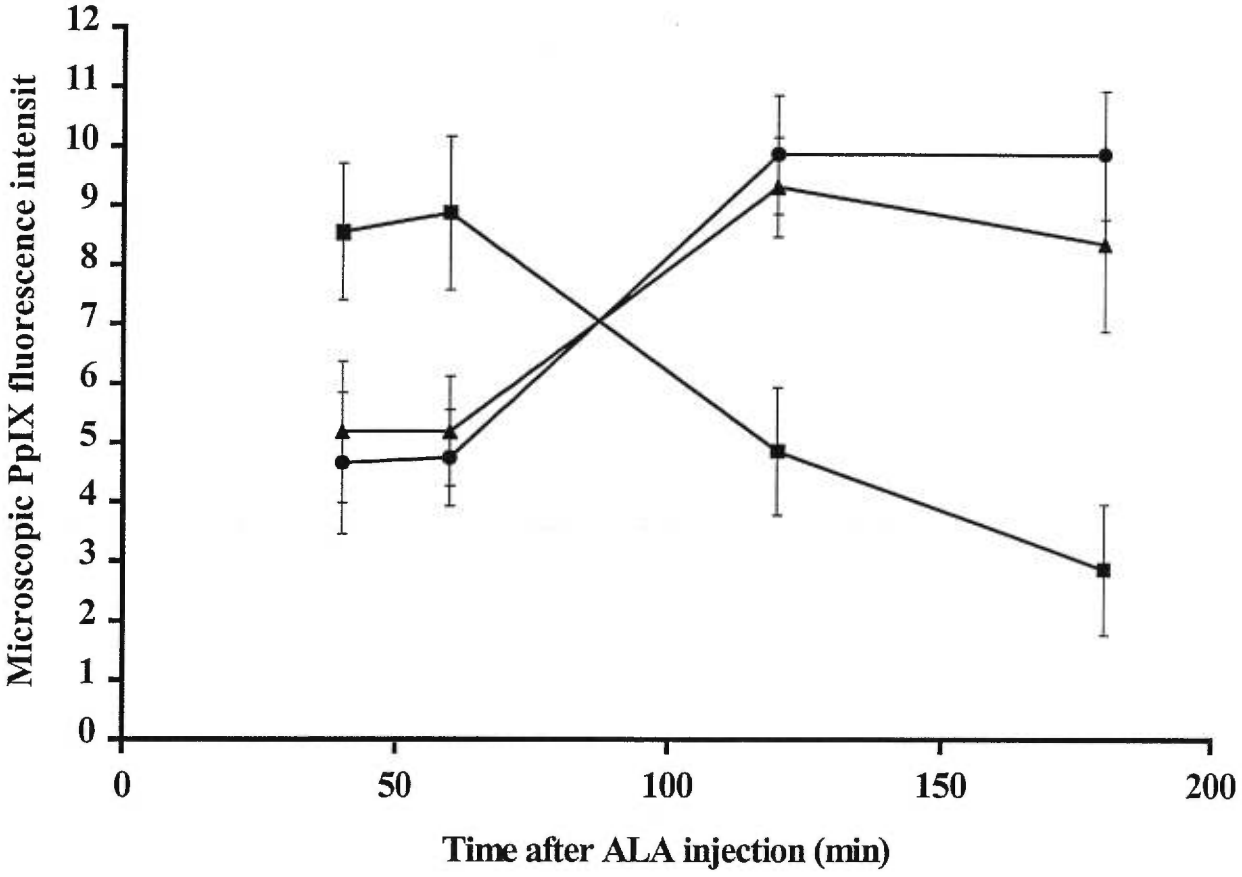


**Figure 2.6. Microscopic protoporphyrin IX fluorescence is higher in tumors than normal and UV-exposed skin 40 minutes after IP ALA at 40 mg/kg ALA.** PpIX red fluorescence intensity after IP ALA at 40 mg/kg as measured by fluorescence microscopy for UV-exposed skin (▲), Small tumor (■), Normal back skin (●).The data represent mean values (± SD).

# Chapter III

## Discussion and Conclusion

## 3.1 Hairless mouse model for UV-induced skin cancer

The process of UV-induced carcinogenesis has been extensively studied in the immunocompetent hairless (Skh:hr1) mouse (de Gruijl and Forbes, 1995). Previous experiments with these mice have yielded quantitative data on the relationship between tumor development and total light dose, time and wavelength of the UV radiation (de Gruijl and Forbes, 1995). When hairless mice are exposed daily to UVB, they eventually all develop skin tumors within 2-6 months after the start of UV exposure (Figure 3.1) (de Gruijl, 1995). In our study, all mice in UV group developed tumors about 4 months after UV irradiation, which is consistent with the previous studies.

De Gruijl and co-workers reported that most UV induced tumors in hairless mice are either actinic keratoses or squamous cell carcinoma (De Gruijl *et al.*, 1983). Tumors smaller than 2 mm mainly consist of actinic keratoses whereas tumors more than 2 mm are mainly squamous cell carcinoma. In our study, most tumors of about 1-2 mm in diameter displayed atypic keratinocytes limited to the epithelium without invasion of the basement indicating that the lesions were actinic keratoses. Larger tumors invaded the dermis and were diagnosed as SCC.

The action spectrum for tumor induction with UVB (280-320 nm) is maximum around 293 nm (de Gruijl *et al.*, 1993). UVA (320-400 nm) also induces squamous cell carcinoma in this mouse model, but the rate of tumor induction by UVA radiation rises more slowly with time and accumulated dose (Berg *et al.*, 1993).

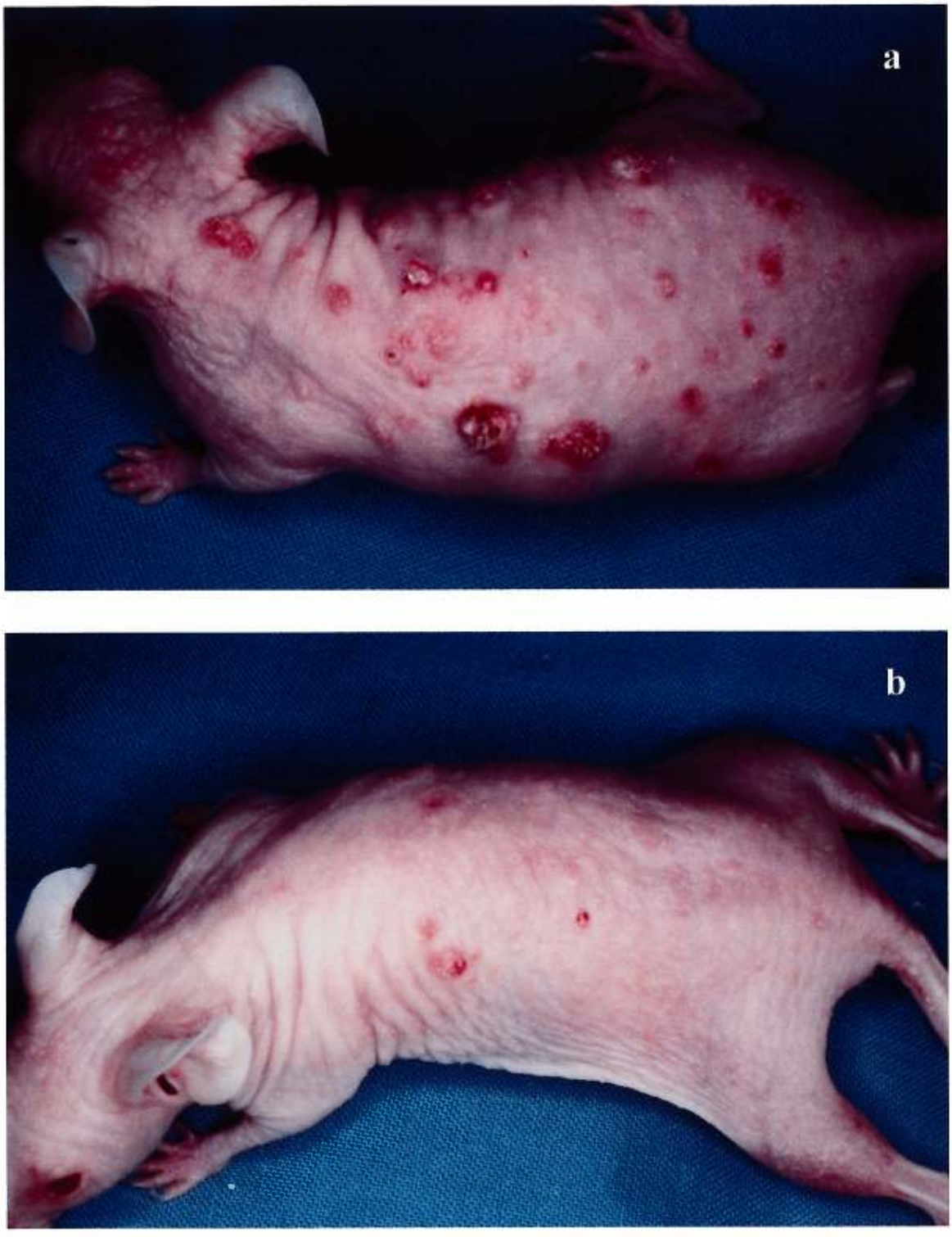


**Figure 3.1. UV-induced skin tumors.** a: a mouse from UV group that was exposed to UV for 22 weeks. b: a mouse from UV/ALA-PDT group that was also exposed to UV for 22 weeks.

## 3.2 Photodynamic therapy with ALA as a mean for prevention of UV-induced skin cancer

Only one other group looked at the ability of photodynamic therapy to prevent UV-induced skin tumors (Stender *et al.*, 1997). Stender *et al.* reported a delay in the appearance of UV induced skin cancer using topical ALA followed by irradiation with visible light after 6-8 hours of ALA application. It has been previously shown that the intensity of PpIX was higher in UV-induced skin tumors as compared with normal skin at 6 hours after the application of topical ALA (Van der Veen *et al.*, 1996).

In this experiment mice developed their first tumor at 21 and 24 weeks after UV irradiation in treated groups with UV and with ALA-PDT respectively. Our tumors were developed earlier than in Stender's study, possibly because they used a different source of UV radiation. They also reported a delay for development of the second tumor in the ALA-PDT-treated group. However, in UV exposed mice that received topical ALA-PDT, mice, had a tendency to develop more large tumors as compared to control groups. They also observed an increase in mortality in the ALA-PDT treated group.

It is not easy to compare Stender's results with our study. In their study, ALA-PDT was performed more aggressively with mice having obvious pain during light exposure and erythema and edema after light irradiation. In contrast, we used the highest dose of light that didn't induce pain or erythema in mice. However, mice became more and more irritable to ALA-PDT as the experiment progressed in both studies.

With systemic ALA, we did not observe the increased incidence of larger tumor reported by Stender. One possible explanation for the higher incidence of large tumor in Stender's study could be the heterogeneity of PpIX fluorescence within tumors. This may lead to poor access of ALA to some malignant cells, resulting in resistance to therapy for some of these cells. Systemic administration theoretically overcomes the problem of limited penetration associated with topical ALA. To prevent UV-induced skin cancer, systemic ALA-PDT may be more practical than topical ALA because repeated whole body topical application is difficult to realize.

### 3.3 ALA-derived PpIX fluorescence for estimation of optimal time for light activation and prediction of PDT response

The tumor selective potential of ALA-derived PpIX and its clinical value for photodynamic treatment of neoplastic lesions have been extensively studied (Bedwell *et al.*, 1992; Henderson *et al.*, 1995; Hua *et al.*, 1995; Orenstein *et al.*, 1996; Peng *et al.*, 1992; Van der Veen, *et al.*, 1996; van der Veen *et al.*, 1994). Considering the variations in the time of maximum PpIX fluorescence intensity observed in this study, quantitative fluorescence measurements of each tissue prior to PDT with ALA could yield valuable information about porphyrin accumulation which is important in order to avoid therapeutic failure of this promising therapeutic technique. The determination of the exact time course of porphyrin fluorescence in tumors may help determining the highest fluorescence intensity in neoplastic tissue and the optimal tumor-host tissue fluorescence ratio (Loh *et al.*, 1993b).

ALA-induced PpIX tumor fluorescence has been reported to increase faster and decay earlier than in normal tissue (Abels *et al.*, 1997; Abels *et al.*, 1994; Van der Veen, *et al.*, 1996; van der Veen, *et al.*, 1994). The earlier PpIX fluorescence peak we observed in skin tumor as opposed to normal skin after ALA administration has also been described for other malignant neoplasms (Abels, *et al.*, 1994; Bedwell, *et al.*, 1992; Chang *et al.*, 1996; Heyerdahl *et al.*, 1997; Leveckis *et al.*, 1994). It has been suggested that the earlier appearance of fluorescence in tumors may be the result of a faster exchange of ALA from the intravascular into the interstitial space and consequently earlier uptake into tumor cells than in host cells (Henderson, *et al.*, 1995). Another factor that also may explain the higher rate of fluorescence increase in tumor as compared with normal skin is an altered activity of enzymes involved in heme biosynthesis in tumors (Van Hillegersberg *et al.*, 1992). It should be noted that selective formation of PpIX in tumors is not only due to reduced ferrochelatase activity but also to reduce intracellular iron levels due to competition for iron between heme production and cellular growth processes (Rittenhouse-Diakun *et al.*, 1995; Van Hillegersberg, *et al.*, 1992).

The maximal concentrations of PpIX in implanted tumors peak around 1-6 h after systemic ALA administration (Bedwell, *et al.*, 1992; Henderson, *et al.*, 1995; Hua, *et al.*, 1995; Orenstein, *et al.*, 1996; Peng, *et al.*, 1992; van der Veen, *et al.*, 1994). The kinetic pattern of ALA-derived PpIX probably depends upon the tumor model and the ALA dose administered. It is noteworthy that tumor porphyrin concentration increased in an ALA dose-dependent manner (Abels, *et al.*, 1997; Regula *et al.*, 1995; van der Veen, *et al.*,

1994). Several studies (Abels, *et al.*, 1997; Regula, *et al.*, 1995; van der Veen, *et al.*, 1994) show dose-dependent tumor and normal tissue PpIX fluorescence.

Abels *et al.* have reported (Abels, *et al.*, 1994) that the time of maximal PpIX fluorescence intensity in tumors was dependent on the ALA dose administered to hamsters implanted with amelanotic melanoma. The maximal PpIX intensity occurred 120 min after administration of 100 mg/kg ALA and 150 min after administration of 500 or 1000 mg/kg ALA (Abels, *et al.*, 1994). Fluorescence in the surrounding host tissue was far less than in the tumor, reaching a maximum 240 min after administration of ALA at 100 mg/kg and 360 min after 500 and 1000 mg/kg ALA. The maximal tumor : host fluorescence ratio was found at 60 min for 100 mg/kg, at 90 min for 500 mg/kg and at 120 min for 1000 mg/kg ALA) (Abels, *et al.*, 1994). Ratios obtained after 100 mg/kg, 500 mg/kg, and 1000 mg/kg ALA were 12 : 1, 90 : 1, and 78 : 1 respectively. In our study, the maximum fluorescence in tumor occurred at 90 min after 40 mg/kg ALA with the highest tumor to normal skin fluorescence ratio occuring 40 min of ALA injection. In another study by Abels *et al* (Abels, *et al.*, 1997), on hamsters implanted with amelanotic melanoma, PDT was performed at the time of maximum fluorescence intensity (150 min with a tumor to host tissue fluorescence ratio of 60 : 1), at 90 min (highest tumor to surrounding host tissue fluorescence ratio with 90 : 1), at 45 min (with a tumor : normal host tissue fluorescence ratio of 30 : 1), and at 300 min (with a tumor to normal tissue PpIX fluorescence ratio of 1) after administration of 500 mg/kg ALA. There was a significant delay in tumor growth after PDT when irradiation was performed 90 min or 150 min. There was no significant difference between these two groups. Irradiation 45

min after injection yielded a smaller, but still significant, delay in tumor growth compared with untreated tumors. There was no significant delay in the tumor volume in group that was exposed to light after 300 min of ALA administration.

In hairless mice, Van der Veen *et al.* (Van der Veen, *et al.*, 1996) have shown that after 200 mg/kg of intraperitoneal ALA PpIX levels were higher in UV-induced squamous cell carcinomas than normal skin (1.8 : 1) after 2 hours of ALA administration. In another study in rat mammary tumor, Van der Veen and co-workers (van der Veen, *et al.*, 1994) found that the maximum tumor and skin PpIX fluorescence ratio following 200 mg/kg ALA was twice as high as after 100 mg/kg ALA, but the initial increase with time was the same during the first 90 min after ALA administration for the two doses in both tumor and skin (van der Veen, *et al.*, 1994). The fluorescence intensity in the 100 mg/kg group reached a peak at 150 min post injection with the optimal tumor fluorescence to normal tissue fluorescence ratio at 120 min after ALA injection. For the 200 mg/kg ALA group, maximum fluorescence intensity occurred 240 min after ALA administration with the maximum tumor:host tissue ratio at 150 min post ALA administration. Vascular effects following ALA-PDT was also examined for different ALA drug dose (100 and 200 mg/kg) at the maximum difference between tumor and normal tissue (120 min for 100 mg/kg and 150 min for 200 mg/kg). The vascular circulation of tumor and normal tissue was observed during and after PDT in anaesthetized animals placed on a temprature-controled stage of microscope. In both groups studied no distinct selective circulation damage of tumor during and after treatment was observed. Furthermore, the overall circulation damage effects were relatively minor, and there was no complete circulation stop in tumor. Despite differences in maximal fluorescence intensity at the

time of light exposure for animals injected with 100 mg/kg and 200 mg/kg, no significant difference in circulation damage during and after PDT was observed. Peng *et al.* (Peng, *et al.*, 1992) observed that PpIX levels were highest at 1 h in mice bearing mammary carcinoma, whereas maximal skin fluorescence was observed at 3 h after intrapretoneal (IP) injection of 250 mg/kg ALA. They also observed a significant delay of tumor growth when PDT was perform at 1 h (maximal fluorescence), but no delay in tumor growth when PDT was performed at 5h and 7h. They concluded that the effect of PDT on tumors decreases when the light exposure is delayed from 1 to 7 h after injection of 250 mg/kg ALA and it is correlated well with the absolute amount of ALA-induce porphyrins in the tumor tissues at different times after systemic injection of ALA.

In our study, the maximum fluorescence intensity in tumor as measured with *in vivo* fluorescence spectroscopy occurred at 90 min after 40 mg/kg of IP ALA whereas the maximum in normal skin occurred 3 hours after ALA administration. Because fluorescence increased earlier in tumor as compared to normal skin, maximum tumor to normal tissue fluorescence ratio occurred after 40 min of ALA injection, before fluorescence peak in tumor.

Transplantable tumors in inbred mice and rats were used in all these studies (Abels, *et al.*, 1997; Abels, *et al.*, 1994; Bedwell, *et al.*, 1992; Orth, *et al.*, 1994; Peng, *et al.*, 1992; van der Veen, *et al.*, 1994) except Van der Veen *et al.* study (Van der Veen, *et al.*, 1996), which used UV-induced skin tumors. These implanted tumors are rarely transplanted into the tissue of origin and the lesions are usually well demarcated and

mostly not invasive, in contrast to UV-induced skin tumors. However, in all studies, a similar distribution / elimination pattern of ALA-induced PpIX was observed in the tumor and normal host tissue. An interesting observation in all studies including ours is the difference between tumor and normal host tissue in the time required to reach maximal fluorescence. Maximal tumor fluorescence was reached earlier than maximal normal tissue fluorescence and as a result, normal skin fluorescence was still increasing at a point when tumor fluorescence had already decreased.

The tumor : normal tissue PpIX fluorescence selectivity appears to be enhanced by using a higher dose for systemic ALA (Loh *et al.*, 1993a; Abels, *et al.*, 1997; Abels, *et al.*, 1994; Regula, *et al.*, 1995; van der Veen, *et al.*, 1994). Abels *et al.* have shown that the optimal ratio of tumor to normal tissue fluorescence increased with increasing ALA dose from 100 to 500 mg/kg (12 : 1 for 100 mg/kg and 90 : 1 for 500 mg/kg ALA). Using mice with implanted colon carcinoma, Henderson and co-workers (Henderson, *et al.*, 1995) reported that tumor porphyrin levels showed pronounced ALA dose dependence after systemic administration of ALA, while normal skin levels showed minimal or no dose dependence. Loh *et al.* have reported that peak levels of fluorescence were achieved earlier with lower doses of systemic ALA (Loh, *et al.*, 1993a). Moreover, the selective accumulation of PpIX in tumors may be enhanced by fractionating ALA administration (Hua, *et al.*, 1995; Regula *et al.*, 1994; Van Hillegersberg, *et al.*, 1992). Regula *et al* (Regula, *et al.*, 1994) found that fractionated ALA dose increased tumor to normal tissue ratios of PpIX from 3:1 to 6:1 in a hamster pancreatic carcinoma model. Hua *et al.* (Hua, *et al.*, 1995) have also reported that high levels of tumor protoporphyrin IX were

sustained by administration of two sequential doses of systemic ALA, at 1.5 and 3 hours after ALA administration in transplanted rat mammary adenocarcinomas (Hua, *et al.*, 1995).

After systemic ALA administration, ALA is rapidly cleared from the circulation. Twenty four to 48 hours after intravenous administration of ALA, hardly any fluorescence was detectable in either tumor or the surrounding host tissue (Fukuda *et al.*, 1992; Henderson, *et al.*, 1995; Peng, *et al.*, 1992; Sroka *et al.*, 1996), which is in accordance with reports that cutaneous photosensitivity following systemic ALA administration in patients is restricted to the first 48 hours post-administration (Bissonnette and Lui, 1997).

## 3.4 Conclusion

We have shown that systemic ALA-PDT can delay the appearance of UV-induced skin cancer without increasing mortality or the incidence of large tumors in hairless mice. Repeated ALA-PDT is thus a promising modality for the prevention of skin cancer that deserves further study.

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