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Targetable photoactivatable drugs

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The photodynamic activity of photosensitizer chlorin e_6 and its targeted or nontargeted polymeric derivatives were evaluated on the human hepatocarcinoma cell line PLC/PRF/5 (targeting structure was galactosamine) or on mouse T splenocytes (targeting structures were anti Thy 1.2 antibodies). It was found that the targeted conjugate is up to 500 hundred times more phototoxic than its nontargeted counterpart. Photodynamic activity of polymeric chlorin e_6 targeted with ATS-A (randomly bound antibody) was detected up to the concentration of 1×10^{-6} M (0.65 μg drug/ml), while photodynamic activity of polymeric chlorin e_6 targeted with ATS-C (oriented binding of antibody via their Fc part) was detected up to the concentration of 1×10^{-8} M (0.0065 μg drug/ml). The final photodynamic effect was dependent on the time and temperature of incubation and on the time of irradiation.

1. Introduction

Most cytostatic drugs have toxic side effects. The lack of selectivity is especially pronounced in chemotherapeutic drugs because they interfere with metabolic processes present in most cells, particularly in proliferating ones. Targeting of cytostatic substances to defined cell populations is an old idea initially formulated by Paul Ehrlich.

Biological activities of enzymes, hormones or antibodies as potential targeting structures are induced only after recognition of their specific targets. The capacity of antibodies to destroy tumours by themselves has always been limited and it has often been observed that specific antibodies can enhance tumour growth (enhancement phenomenon). As a result, a series of attempts have been made to render the effector function of antibodies more potent by attaching anticancer agents to these antibodies [1, 2].

The potential for exploiting the high specificity characteristic for antibodies to bestow a high degree of selectivity on indiscriminately lethal drugs has long been an attractive possibility. However, chemical manipulation can both inactivate antibody-combining sites and disturb the integrity of the cytotoxic agents themselves. The construction of targeted immunosuppressants or cytotoxics in general is simple in concept, but a number of requirements must be fulfilled for a conjugate to be of therapeutic value.

In recent years, photodynamic therapy (PDT) has appeared as a relatively new approach for the treatment of different tumours [3–6] and photosensitizing drugs are also used as potent immunosuppressive agents [7–9]. Selective toxicity might be achieved in PDT if a photoactivatable drug is directed towards a particular subset of cells with the aid of an appropriate targeting structure (antibodies, carbohydrates, hormones, lectins) to which specific receptors are expressed on the membrane surface of target cells. By illumination of target area only, two-fold specificity is achieved.

The therapeutic effectiveness of PDT depends upon the relative drug levels in the tumour and the surrounding normal tissues exposed to similar doses of light. To increase the concentration of the photosensitizer in the target tissue and to decrease its binding to non-diseased tissue we have developed two models of PDT which increase the selectivity of the treatment. The photosensitizer, chlorin e_6, was attached to the oligopeptide side sequences of a water-soluble synthetic copolymer carrier based on *N*-(2-hydroxypropyl)methacrylamide (HPMA) [10, 11] to which also targeting moieties, galactosamine or anti Thy 1.2 antibodies, were coupled. HPMA enabled us to increase the drug: targeting ratio; it protects antibodies against inactivation and effectively eliminates the toxic side effects of the drug [12, 13].

The photodynamic activity of a polymeric photosensitizer containing galactosamine was tested against the human hepatocarcinoma cell line PLC/PRF/5 displaying a surface membrane asialoglycoprotein receptor promoting the uptake and endocytosis of glycoproteins. Anti Thy 1.2 targeted polymeric chlorin e_6 was evaluated on mouse splenic T cells expressing Thy 1.2 alloantigen. These two models allowed us to compare the photodynamic abilities of targeted polymeric chlorin e_6 in cancer chemotherapy and as immunosuppressant.

As the reaction conditions, such as temperature and the time of contact of the sensitizer with the cells, can affect the pattern of damage, we have tested the effect of irradiation time, incubation time as well as influence of temperature on the photodynamic activity of the free, targeted or nontargeted polymeric chlorin e_6.

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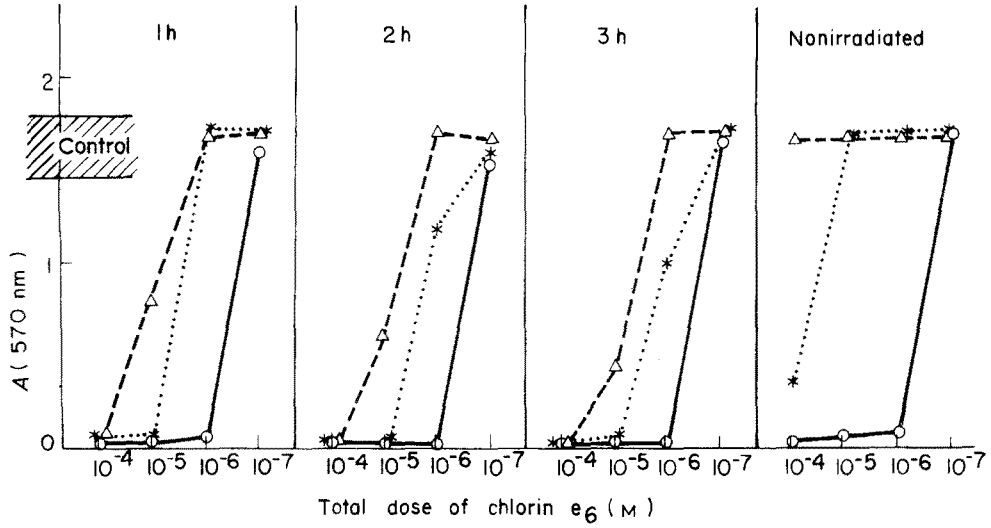

Figure 1 The effect of incubation time (at 37 °C) on the photodynamic destruction of human hepatocarcinoma cell line PLC/PRF/5, incubated with free, polymeric targeted or nontargeted chlorin e_6; irradiation time 7.5 h. (○) Chlorin e_6, (△) P-chlorin e_6, (*) P—chlorin e_6 / Gal

2. Experimental procedure

HPMA copolymers containing galactosamine and chlorin e_6 were prepared according to Krinick *et al.* [14] and those containing anti Thy 1.2 antibodies according to Krinick *et al.* [15]. Two types of anti Thy 1.2 antibody-containing conjugates were synthesized. They differ in the method of antibody binding. The conjugate with ATS-A was synthesized by aminolysis of the polymeric precursor with *N*-(2-aminoethyl)chlorin e_6 amide, followed by aminolysis with anti Thy 1.2 antibodies [15]. In this conjugate the antibodies were bound via N^{ε}-amino groups of lysine residues. The conjugate with ATS-C contained antibodies bound via oxidized carbohydrate groups in the Fc part of the antibody molecule.

Generally, each well in 96-well tissue culture plates was seeded with 1×10^5 hepatocarcinoma cells or with 2×10^6 splenocytes. Cells were incubated (4 or 37 °C) in media without FCS with different samples. After incubation the cells were washed with PBS, resuspended in fresh Iscove's medium, irradiated and incubated (37 °C, 5% CO_2) for 18 h to obtain the maximal phototoxic effect. The viability of splenocytes was estimated using the Trypan Blue exclusion test [16], the viability of hepatocarcinoma cells was tested using the MTT test [17].

3. Results and discussion

3.1. Effect of incubation time

The rate of uptake of a free drug, a polymeric targeted or nontargeted drug, might differ due to different mechanisms by which these molecules enter the cell. To determine the time necessary for efficient loading of the cells with the photosensitizer, the hepatocarcinoma cells or splenocytes were incubated with free, targeted or nontargeted polymeric chlorin e_6 for 1–3 h (Fig. 1, Table I). At all time intervals significant cell death was observed in irradiated as well as in nonirradiated tissue culture when exposed to a free drug up to a concentration of 1×10^{-6} M. The same concentration of polymer-bound chlorin e_6 is toxic only if irradiated.

The toxicity had a delayed character being fully detectable only after 24 h after the last contact with the drug; it was not apparent if the cell viability was determined immediately after incubation. It is not yet clear if this time interval is needed because chlorin e_6 should accumulate in organelles such as mitochondria or lysosomes or if the dark toxicity is due to the long-lasting effect of the hydrophobic photosensitizer on the cell membrane.

The results show that the rate of uptake is considerably influenced by the form and concentration of

TABLE I The effect of time of incubation with free or polymeric targeted or nontargeted chlorin e_6 on the viability of mouse splenocytes[a]

Samples	Concentration chlorin e_6 (10^{-6} M)	Incubation			Nonirradiated
		1 h	2 h	3 h	
P—chlorin e_6 / ATS-A	1	16[b]	15	23	15
P—chlorin e_6 / ATS-C	1	50	60	59	14
P-chlorin e_6	1	16	15	18	13
Chlorin e_6	1	55	65	70	52
Control	–	8	12	10	12

[a] Irradiation for 7.5 h (662 nm).
[b] Numbers represent dead cells (%).

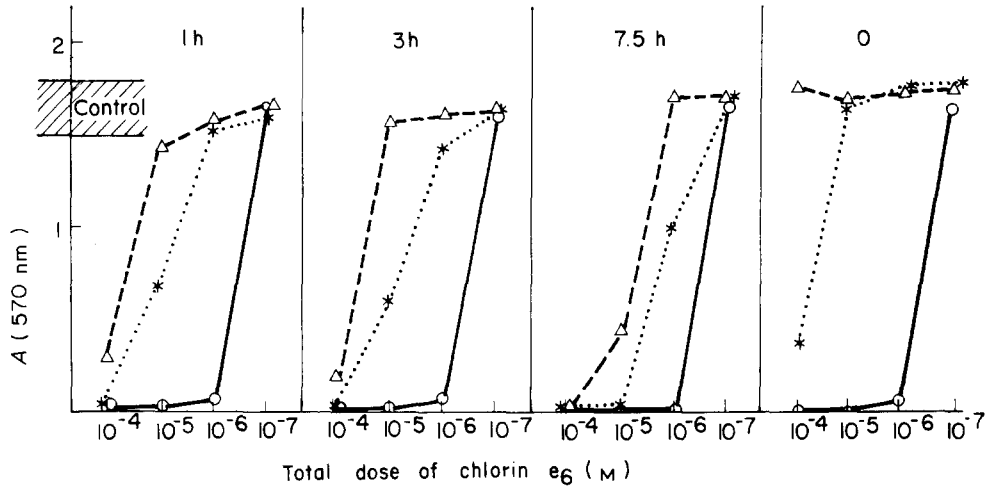

Figure 2 The effect of irradiation time on the photodynamic destruction of human hepatocarcinoma cell line PLC/PRF/5, incubated with free, polymeric nontargeted or targeted chlorin e_6 for 3 h at 37 °C. (○) Chlorin e_6, (△) P-chlorin e_6, (*) P<chlorin e_6 / Gal

the photosensitizer and by the character of the cell surface receptor. Free chlorin e_6 reached a photodynamically effective intracellular concentration in less than 1 h in both systems. Incubation for 1 h was also sufficient to load cells with a lethal concentration of polymeric chlorin e_6 if it was targeted with anti Thy 1.2 antibodies. In the system of the human hepatocarcinoma cell line exposed to polymeric chlorin e_6 targeted with galactosamine, 3 h were needed for significant killing effect (cf. Fig. 1, Table I).

This observation could be a result of several phenomena. Free and polymer-bound drugs enter cells by different mechanisms. The mechanisms of cellular uptake of extracellular molecules include either diffusion across the cell membrane (free drug), endocytosis involving fluid phase pinocytosis (nontargeted polymer-bound drug) or receptor-mediated endocytosis (targeted polymer-bound drug) [18].

The photodynamic activity of the polymeric photosensitizer depends on its binding to cell surface structures. Current experience indicates that for a binding site to be effective in facilitating intoxication, it must promote endocytosis of the drug [19]. Immunotoxins made with antibodies to sites that are efficiently endocytosed are usually much more active than those with antibodies to sites with a low endocytosis rate [20].

In comparing the two different targeted polymer-bound drugs, three things could lead to their differences in cellular uptake: (a) a different number of membrane receptors on the respective cells, (b) different affinity of receptors, (c) different rate of uptake via the asialoglycoprotein receptor or via the Thy 1.2 alloantigen.

3.2. Effect of irradiation time

Unlike incubation, the time of irradiation exhibited a similar dependence in both systems, i.e. in a culture of hepatocarcinoma cells (Fig. 2) and in a culture of mouse T cells (Table II). Irradiation for 1 h led to significant destruction of target cells, but the phototoxicity and cell death increased with the time of irradiation. This dependence on the time of irradiation was not seen in cultures incubated with free drug, where 1 and 7.5 h irradiation induced the same photodynamic effect.

TABLE II The effect of time of irradiation on the viability of mouse splenocytes incubated with free or polymeric targeted or nontargeted chlorin e_6[a]

Samples	Concentration chlorin e_6 (10^{-6} M)	Irradiation			Nonirradiated
		1 h	3 h	7.5 h	
P<chlorin e_6 / ATS-A	1	15[b]	20	23	11
P<chlorin e_6 / ATS-C	1	32	38	59	14
P-chlorin e_6	1	18	15	18	12
Chlorin e_6	1	65	70	70	56
Control	–	9	9	10	11

[a] Incubation at 37 °C, 3 h.
[b] Numbers represent dead cells (%).

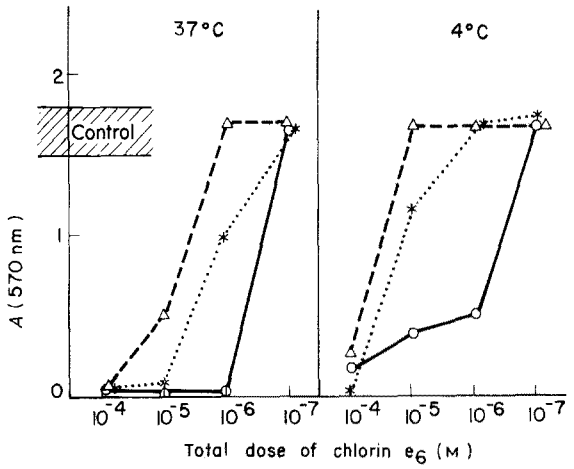

Figure 3 The effect of temperature of incubation ((a) at 37 °C or (b) at 4 °C) on the photodynamic destruction of human hepatocarcinoma cell line PLC/PRF/5. (○) Chlorin e_6, (△) P-chlorin e_6, (*) P<chlorin e_6 / Gal

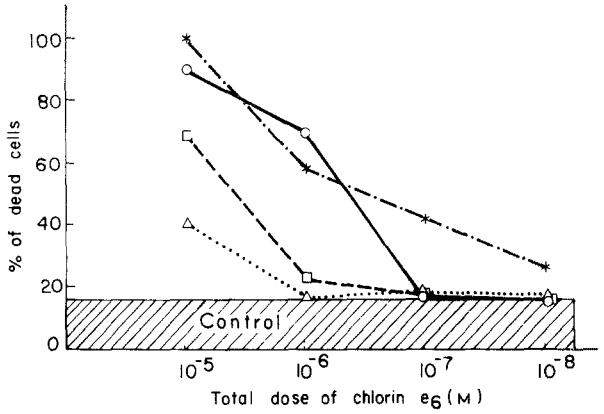

Figure 4 The effect of oriented binding of targeting antibody on photodynamic activity of polymeric chlorin e_6; incubation for 3 h at 37 °C; irradiation time 7.5 h. (○) Chlorin e_6, (△) P-chlorin e_6, (□) P<chlorin e_6 / ATS-A, (*) P<chlorin e_6 / ATS-C

3.3. Effect of temperature

To distinguish between photodestruction caused by the damage of the plasma membrane alone and photodestruction due to intracellularly entrapped drug, the hepatocarcinoma cells were incubated with different samples for 3 h at 37 or 4 °C, because processes such as binding to cell membrane, pinocytosis or receptor-mediated endocytosis have a different temperature dependency [21]. In contrast to the observation made by Oseroff *et al.* [22] we have observed a considerable decrease of phototoxicity (Fig. 3) if binding and irradiation were carried out at 4 °C, compared with the situation at an endocytic permissive temperature (37 °C). These results prove that to be fully phototoxic, chlorin e_6 has to accumulate intracellularly and that an energy-and temperature-dependent mechanism is responsible for transmembrane transport of both the free and the polymeric drug.

3.4. Effect of antibody binding on phototoxic activity

Anti Thy 1.2 antibodies were bound to the copolymer carrier either randomly (aminolysis via N^{ε}-amino groups of a lysine residue; ATS-A) or oriented via an oxidized carbohydrate of the Fc part of antibody molecule (ATS-C). Random or oriented binding causes a different targeting and consequently phototoxic effectiveness to polymeric photosensitizers. Chlorin e_6 targeted with ATS-C is considerably more phototoxic than its counterpart targeted with ATS-A (Fig. 4) and its destruction ability exceeds even the activity of the free drug. A concentration of 1×10^{-7} M ATS-C-targeted photosensitizer kills about 40%, 1×10^{-8} M about 30% of T cells while a free drug at the same concentration is ineffective. These data indicate that oriented binding increases the targeting capacity and thus the phototoxic activity of a polymeric drug.

Nontargeted polymeric chlorin e_6 was less phototoxic in both systems when compared to its targeted counterpart. The photodynamic activity of polymeric chlorin e_6 targeted with ATS-A is comparable with the photodynamic activity of polymeric chlorin e_6 targeted with galactosamine (Figs 5, 6). Phototoxicity was observed up to the concentration of 1×10^{-6} M (0.65 µg drug/ml). Targeting capacity and photodynamic activity of a polymeric prodrug containing ATS-C (binding via the Fc fragment never involves

	Total dose of chlorin e_6 (M)			
Sample	1×10^{-5}	1×10^{-6}	1×10^{-7}	1×10^{-8}
Chlorin e_6	+++	+++	±	0
P<chlorin / ATS A	+++	+	±	0
P<chlorin / ATS C	++++	+++	++	+
P–chlorin	+	±	0	0

Figure 5 Photodynamic effect of free, targeted or nontargeted chlorin e_6 on mouse splenocytes; incubation time 3 h, irradiation time 7.5 h. Percentage of dead cells: (±) 15–25, (+) 25–40, (++) 40–70, (+++) 70–90, (++++) 90–100.

	Total dose of chlorin e_6 (M)			
Sample	1×10^{-4}	1×10^{-5}	1×10^{-6}	1×10^{-7}
Chlorin e_6	+++	+++	+++	0
P<chlorin / Gal	++++	+++	+	0
P–chlorin	+++	+	0	0

Figure 6 Photodynamic effect of free, targeted or nontargeted chlorin e_6 on human hepatocarcinoma cell line PLC/PRF/5. Incubation time 3 h, irradiation time 7.5 h. A = adsorbance at 570 nm: (0) ≥ 1.15, (+) 0.5–1.15, (++) 0.1–0.5, (+++) 0.01–0.1, (++++) 0.0.

the antibody binding sites) is considerably higher. Phototoxicity was observed up to the concentration 1×10^{-8} M (0.0065 μg chlorin/ml).

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