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Biodistribution and toxicity of photoproducts of merocyanine 540

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Summary. Light-activated merocyanine 540 (pMC540) has been shown in our earlier studies to be effective against certain types of tumor cells and viruses, including human immunodeficiency virus (HIV-1). To test the potential extracorporeal and systemic use of pMC540, its toxicity was investigated in DBA/2 mice, pigs, and dogs. The lethal dose in DBA/2 mice after an i.p. injection was 370 mg/kg, and the 50% lethal dose (LD_{50}) was 320 mg/kg; however, following i.v. administration, the lethal dose and the LD_{50} dose were 240 and 160 mg/kg, respectively. Tritium-labeled MC540 was used to study the biodistribution of pMC540 in DBA/2 mice. Almost 70% of the injected radioactivity was excreted within 6 h of injection. After 1 week, the pMC540 was almost completely cleared, with only 1.89% of the activity remaining, and had a plasma half-life of 23 h. Pigs injected with an accumulated dose of 10 mg/kg and followed for a period of 30 days did not show adverse signs of toxicity as monitored by SMAC-28 analysis, CBC profile, and blood-coagulation studies. A dog injected with a single dose of 20 mg/kg showed induction of the hepatic enzymes glutamic oxaloacetic transaminase (AST) and glutamic pyruvic transaminase (AST); however, serum levels of gamma-glutamyl transpeptidase (GGT) remained unchanged. The data presented herein may serve to identify certain drug-dose limitations in the systemic use of pMC540.

Introduction

The potential of various photoactive compounds as antiseptic, antibacterial, antiviral, and antitumor agents is well established. The ability of these compounds to become excited on exposure to light has been exploited in vitro and in vivo [2, 4, 9, 19, 22]. However, simultaneous exposure of the target to the photoactive agent and to light is a prerequisite for photodynamic action, thereby limiting the application of photodynamic therapy to accessible targets, e.g., superficial skin lesions or localized solid tumors to which the light could easily be delivered. To overcome this limitation, we have described a novel process termed "preactivation" [3, 12, 13].

The process of preactivation involves controlled exposure of photoactive compounds to light *prior to* their use in biological targets, resulting in the formation of heretofore unknown photoproducts. The biological activity of preactivated merocyanine 540 (pMC540) has been reported. The results of these studies showed that pMC540 was cytotoxic to certain tumor cell lines and enveloped viruses (herpes simplex virus I, simian immunodeficiency virus, and human immunodeficiency virus I); the photoactivated compound retained its antitumor and antiviral activity over a period of at least 30 days at –135° C [3, 12, 13]. Data also indicated that pMC540 displays target-preference, i.e., it showed minimal toxicity toward normal hematopoietic cells [13]. The present study was carried out to evaluate the in vivo toxicity of pMC540. We report the results of investigations conducted to study the toxicity, biodistribution, and plasma half-life of pMC540 in animal models.

Materials and methods

Merocyanine 540. MC540 was obtained from Sigma Chemical Co. (St. Louis, Mo.). The concentration of the stock solution was 1 mg/ml in 10% ethanol: water (v/v). Tritium labeling was performed by New England Nuclear, Inc. (Boston, Mass.). The precursor, MC540 (200 mg), was dissolved in 4 ml dimethylformamide. To this was added 50 mg 5% RL/Al_2O_3 and 25 Ci tritiated water. The reaction mixture was stirred overnight at 50° C. Labile components were removed with ethanol, and the purity was checked by comparative high-performance liquid chromatographic (HPLC) analysis of unlabeled and labeled material. The elution profile of labeled MC540 injected onto a reverse-phase C_{18} column and eluted with 95% ethanol displayed 98% homology in the

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Abbreviations: pMC540; p-^{3}H-MC540; AST; ALT; CPK; GGT

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area of the peak as compared with the unlabeled material. The final stock was made in ethanol : water (1 : 1, v/v) at 5 mg/ml. The stock solution of [^{3}H]-MC540 (specific activity, 0.98 μCi/mg) was provided as a 5-mg/ml solution in 50% ethanol : water (v/v). Small aliquots of MC540 and [^{3}H]-MC540 were stored in a dark environment at –20° C.

Light activation. Photoactivation of MC540 and [^{3}H]-MC540 was accomplished by exposing 1 mg MC540/ml and 150 or 240 μCi (for biodistribution studies) [^{3}H]-MC540/ml in 2.5% aqueous ethanol to light from eight fluorescent lamps (Philips cool white, 20 W) held at a 10-cm distance from the samples for 18 h [12]. The cytotoxicity of photoactivated [^{3}H]-MC540 (p-[^{3}H]-MC540) against Daudi cells (in-house standard) was virtually identical to that of pMC540, and the absorption maxima at 280 nm for p-[^{3}H]-MC540 and pMC540 were comparable, indicating that the photoactivation process occurred appropriately as described previously [12, 13]. Activated solutions were stored in a dark environment at –135° C and were used within 15 days of preparation.

Animals. Female DBA/2 mice (6–8 weeks old; weighing 20–25 g each) were obtained from Charles River Co. (Boston, Mass.). Animals were fed ad libitum and had free access to drinking water. Pigs (male white breeds, Hampshire crosses) weighing approximately 20 kg each were obtained from the Ken Watterson farm (Dallas, Texas). A 6-year-old male dog (crossbred mongrel) was injected with a single dose of 20 mg/kg pMC540 through the cephalic vein.

Biodistribution. For biodistribution studies, p-[^{3}H]-MC540 was injected i.p. into DBA/2 mice. Three separate sets of experiments (n = 3 animals/set) were done for each time point. The total dose given was 80 μCi/mouse in a final volume of 330 μl. Injected mice were kept in individual metabolic cages. Urine and fecal samples were collected from the "catch pans" of the cages. Animals were killed by cervical dislocation at various time points (between 2 h and 1 week), and autopsies were performed. All vital organs were removed, homogenized separately, solubilized in Soluene 350 tissue solubilizer (Packard Instrument Company, Inc.) for 24–48 h, and decolorized with 30% hydrogen peroxide to reduce quenching [19]. Samples were then dissolved in toluene-based scintillation cocktail (containing 2,5-diphenyloxazole and 2,2′-*p*-phenylene-bis[5-phenyloxazole]). The radioactivity in each dissolved whole organ from each animal was measured separately using a model LS 1701 Beckman scintillation counter (Beckman Instruments, Nuclear Systems Operations, Fullerton, Calif.).

Plasma half-life. The plasma half-life of p-[^{3}H]-MC540 was determined following an i.p. injection of 50 μCi (50 μg) in a final volume of 330 μl into DBA/2 mice. For each time point, three separate sets of experiments were done (n = 4/set). Blood samples were obtained from the heart at various time points. Heparin was used as an anticoagulant. These samples were diluted with phosphate-buffered saline (pH = 7.4) to reduce color quenching by plasma (the quenching caused by phosphate-buffered saline was less than that produced by plasma alone). The diluted blood samples were centrifuged at 1,500 *g* for 10 min to pellet the cellular component. An aliquot from the supernatant was counted in 10 ml scintillation cocktail. Counts per minute (CPM) were adjusted for the total volume and converted to into units of decay per minute (DPM). DPM were converted to into nanograms of p-[^{3}H]-MC540 per milliliter from the average DPM (115,190,150) obtained from 50 μCi. The plasma half-life of p-[^{3}H]-MC540 was calculated by nonlinear regression analysis.

In vivo toxicity. pMC540 was evaluated for its toxicity in three animal species. Three separate sets of experiments were performed on DBA/2 mice (n = 9 per set per dose) for determinations of the lethal dose (LD_{100}) and the 50% toxic dose (LD_{50}) of pMC540. The dose range used was 10–240 mg/kg. The volume of compound injected was 250–300 μl. Mice were given i.p. injections of pMC540 and were followed for a period of 10 days. In a separate set of experiments, the lethal dose and the LD_{50} were determined following i.v. administration of pMC540 via the tail veins of the mice (n = 9). A control group (n = 9) of animals were injected with an equal volume of the drug solvent (2.5% ethanol : saline).

For evaluation of the effect of pMC540 on hematologic functions over a period of 30 days, larger animals (pigs and dogs) were used. The acute toxicity studies in mice suggested that these animals tolerated a dose of 100 mg/kg without developing any apparent side effects. It has been reported that in clinical trials, the treatment dose routinely selected for human use is 1/100th to 1/10th of the minimal effective dose [14]. In view of this information and in consideration for the safety of the animals, a dose of 10 mg/kg was chosen for the first large animal group receiving the drug. Pigs (n = 3) were injected with pMC540 (accumulated dose, 10 mg/kg) in 50 ml 2.5% aqueous ethanol in 0.9% saline solution delivered through an indwelling surgically implanted subclavian catheter passed into animals under enflurane inhalation anesthesia (via endotracheal intubation). The anesthesia was maintained at 2% enflurane and 2% O_2 using a flow rate of 500 ml/min. Following catheterization, the cannula was secured by skin stitches and kept patent by daily flushing with heparin. Immediately after catheterization, a baseline blood sample was obtained from the animals. The dosing regimen was 2 mg/kg pMC540 on day 0, 3 mg/kg on day 3, and 5 mg/kg on day 7, the drug being given through the catheter after the animals had been tranquilized with an i.m. injection of Valium (10 mg) or ketamine (5 mg). This dosing regimen was adopted to enhance the safety factor.

As adverse effects were not observed at a cumulative dose of 10 mg/kg, a higher dose of 20 mg/kg was chosen for studies in dogs to determine the single-dose toxicity. pMC540 was given through the cephalic vein of the dogs. The composition of the dosing solution was 2.5% aqueous ethanol in 0.9% saline infused at a rate of approximately 100 ml/h. In each set of experiments, control animals received 50 ml 0.9% saline solution. Blood samples from all animals participating in the study were collected on days 1, 3, 5, 7, 14, 21, and 28 for various laboratory profiles. Animals were killed after 30 days with 8 mg TGI euthanasia solution, autopsies were performed, and organs were removed for histopathologic examination.

Results

In vivo toxicity and biodistribution

Table 1 shows the radioactivity recovered following the administration of p-[^{3}H]-MC540 (i.p. to DBA/2 mice. At 2 and 4 h after the injection of the drug, none of the major organs showed a preferential accumulation of radioactivity. The data show that the majority of the radioactivity was excreted from the animals' body within 6 h. The brain, liver, and kidneys constituted the main organs accounting for the remaining activity. By 48 h following injection, only 21% of the remaining activity had been recovered, with the bulk accumulating in the skin, muscles, and bone (>15%). At 1 week after the injection, p-[^{3}H]-MC540 was almost completely cleared, with only 1.89% of the activity being recovered. It should be noted that in Table 1, subsequent to the 6-h time point, the urine and feces data, which amount to 70% of the radioactivity, are not reflected in the total amount recovered. Altogether, the total drug elimination accounts for >90% of the radioactivity recovered.

The data were converted into the amount of radioactivity recovered per gram of wet organ weight. The results show that 48.84% of the radioactivity was recovered from the brain at 6 h after the injection (Table 2). The kidneys accounted for 22.93% of the retained radioactivity, followed by the spleen (10.86%) and the heart (7.52%). The percentage of distribution per gram of tissue began to decline (38%) in the brain at 24 h after injection; however, the latter nonetheless constituted the major organ in which

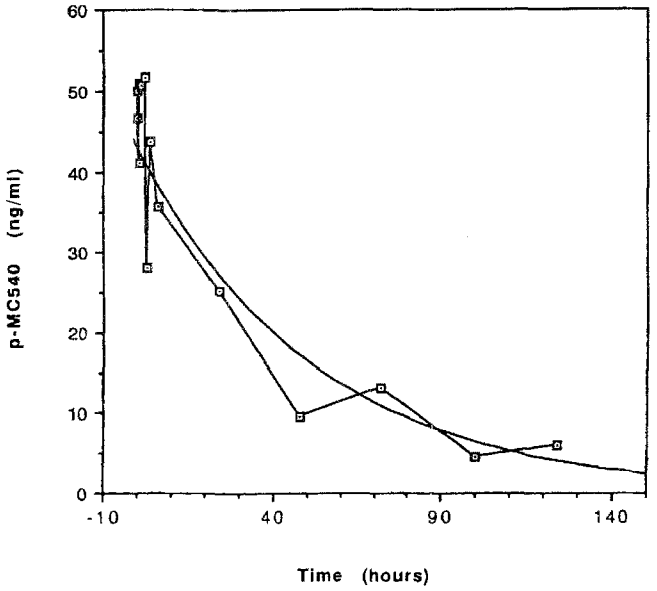

Fig. 1. Plasma clearance of p-[^{3}H]-MC540 was determined by an intraperitoneal injection of 50 μCi (50 μg) into DBA/2 mice. Blood samples were obtained at various time points. These samples were diluted with PBS (pH 7.4) and centrifuged at 1500 *g* for 10 min to pellet the cellular component. An aliquot from the supernatant was taken in 10 ml scintillation cocktail, and radioactivity was counted using a model LS 1701 Beckman scintillation counter. Radioactivity counts were converted into ng p-[^{3}H]-MC540/ml from the average DPM obtained from 50 μCi

the radioactivity resided after 24 h (Table 3). An increased distribution of p-[^{3}H]-MC540 in the spleen (21.12%) was observed at 24 h postinjection. Distribution was also increased in the heart tissue (12.71%) at this time point. The maximal radioactivity at 48 h after injection (Table 4) was recovered from the kidneys (25.19%), followed by the lungs (21.37%), heart (17.98%), spleen (16.52%), and brain (16.27%). Accumulation of radioactivity was also observed in the muscle tissues (4.49%) and the skin (2.87%) at 48 h after injection. By 96 h, the radioactivity had become maximal in the muscle tissues (30.29%) and lungs (24.79%; Table 5). The hepatic distribution of pMC540 appeared to be relatively low, with only 3.87% of the recovered radioactivity being observed at 6 h after injection, reaching its peak (4.16%) within 24 h and subsequently falling to only 1.06% of the total recovered activity at 96 h following injection.

The plasma half-life of p-[^{3}H]-MC540 was evaluated after a single i.p. injection of 50 μg (50 μCi) into DBA/2 mice. Blood samples were obtained at various time points, and the activity in the plasma was monitored. The radioactivity recovered was converted (as described in Materials and methods) into nanograms of the drug per milliliter and was plotted against time as shown in Fig. 1. The data show that the maximal concentration of the drug in the plasma was obtained at 2 h after an i.p. injection. This concentration was 51.68 ng/ml plasma. The results show that at 24 h after the injection, the plasma-drug concentration was very close to the half-maximal drug levels, i.e., 25.13 ng/ml. The plasma half-life ($t_{1/2}$) of p-[^{3}H]-MC540 was calculated

Table 1. Percentage of distribution of p-[^{3}H]-MC540[a]

Samples	Percentage of injected radioactivity						
	2 h	4 h	6 h	24 h	48 h	96 h	1 week
Liver	0.09	0.12	4.35	3.59	0.86	0.21	0.14
Kidney	0.07	0.06	4.49	2.64	1.19	0.31	0.21
Spleen	0.03	0.01	1.37	1.51	0.34	0.21	0.17
Heart	0.01	0.00	1.59	1.69	0.74	0.34	0.24
Brain	0.07	0.06	5.35	6.67	0.31	0.69	0.39
Lungs	0.02	0.01	1.06	0.94	0.97	0.81	0.34
Urine	ND	47.00	42.00	3.10	0.59	0.09	0.05
Feces	ND	33.00	28.00	2.74	0.67	0.11	0.06
Others	ND	19.71	11.79	7.11	15.31	1.27	0.28

[a] Light-activated [^{3}H]-MC540

Data represent mean values for 4 separate sets of experiments consisting of 2–3 animals/group. The radioactivity values in urine and feces, accounting for approximately 70%, are not reflected in the amount of radioactivity recovered subsequent to 6-h time points. ND, Not determined

Table 2. Biodistribution of p-[^{3}H]-MC540[a] in DBA-2 mice at 6 h after an intraperitoneal injection

Organ	Wet weight (g)	DPM/g	% Distribution
Liver	0.935±0.065	408,273± 322,728	3.87±0.49
Kidney	0.170±0.026	2,398,777± 761,950	22.93±6.55
Spleen	0.111±0.034	1,156,664± 379,357	10.86±2.57
Heart	0.177±0.003	788,806± 153,454	7.52±1.89
Brain	0.094±0.025	5,202,204±1,164,645	48.83±7.95
Lungs	0.147±0.007	629,905± 32,451	5.97±0.54

[a] Light-activated [^{3}H]-MC540

Table 3. Biodistribution of p-[^{3}H]-MC540[a] in DBA-2 mice at 24 h after an intraperitoneal injection

Organ	Wet weight (g)	DPM/g	% Distribution
Liver	0.825±0.09	383,378± 34,455	4.16±0.28
Kidney	0.136±0.026	1,701,904±107,221	18.49±0.23
Lung	0.155±0.009	530,902± 54,258	5.76±0.30
Heart	0.126±0.024	148,025± 24,832	12.71±1.30
Brain	0.168±0.003	3,474,838±377,409	37.75±3.33
Spleen	0.07 ±0.02	1,940,739±363,243	21.12±3.99

[a] Light-activated [^{3}H]-MC540

Table 4. Biodistribution of p-[^{3}H]-MC540[a] in DBA-2 mice at 48 h after an intraperitoneal injection

Organ	Wet weight (g)	DPM/g	% Distribution
Liver	0.968±0.046	78,248±20,736	2.66±0.62
Kidney	0.142±0.003	733,810±73,190	25.19±3.16
Heart	0.123±0.003	525,582±50,902	17.98±1.33
Lung	0.136±0.004	623,202±62,025	21.37±2.36
Brain	0.058±0.002	475,346±58,838	16.27±1.76
Spleen	0.062±0.006	484,104±85,029	16.52±2.34

[a] Light-activated [^{3}H]-MC540

Table 5. Biodistribution of p-[^{3}H]-MC540[a] in DBA-2 mice at 96 h after an intraperitoneal injection

Organ	Wet weight (g)	DPM/g	% Distribution
Lungs	0.167±0.015	430,758± 124,437	24.79±5.93
Muscle	0.122±0.007	522,189± 22,490	30.29±1.46
Skin	0.314±0.031	50,117± 10,766	2.89±0.55
Brain	0.240±0.023	251,115± 65,355	14.55±3.59
Liver	1.002±0.042	18,480± 3,745	1.06±0.16
Kidney	0.426±0.021	64,107± 3,073	3.71±0.09
Spleen	0.102±0.005	183,829± 14,992	10.72±1.68
Heart	0.146±0.004	206,169± 13,193	11.94±0.28

[a] Light-activated [^{3}H]-MC540

by nonlinear regression analysis and determined to be 23.1 h.

The in vivo toxicity of pMC540 was determined in three different animal species, namely, mice, dogs, and pigs. The LD_{100} in DBA/2 mice after i.p. injection was 370 mg/kg, and the LD_{50} was 320 mg/kg. In a separate set of experiments, the LD_{100} and LD_{50} doses were determined after i.v. administration of pMC540 and were found to be 240 and 160 mg/kg, respectively (data not shown).

Pigs were injected with escalating doses of pMC540 through an indwelling, surgically implanted subclavian catheter. Blood samples were taken every 24 h for a period of 1 week and then weekly for 30 days after pMC540 administration. Samples were subjected to SMAC-28 analysis and complete blood profile. Pigs injected with escalating doses of pMC540 showed no abnormality in terms of serum electrolytes, hepatic functions, renal functions, or cardiac enzymes as shown in Tables 6 and 7. An increase in creatinine phosphokinase (CPK) activity was observed immediately after the first injection; however, this rise was transient, as values returned to baseline levels by day 3. This rise in CPK activity may have resulted from the animals, having being given an i.m. injection of Valium to restrain them prior to drug injection. CPK levels are known to increase following skeletal or cardiac (but not smooth) muscle damage. No abnormality was observed in the blood profile as seen in CBC analysis, and blood-coagulation factors were within the normal range (Table 6).

Table 6. In vivo toxicity of p-MC540* in pigs

Laboratory test	Baseline	Day 0 2 mg/kg Injection	Day 1	Day 3 3 mg/kg Injection	Day 5	Day 7 5 mg/kg Injection	Day 15	Day 30
SMAC-28								
Glucose	96.0	111.0	105.0	108.0	139.0	130.0	128.0	114.0
Sodium	139.0	143.0	142.0	147.0	143.0	142.0	144.0	140.0
Potassium	3.7	3.8	4.1	4.4	4.5	4.0	4.3	3.8
Chloride	100.0	103.0	103.0	105.0	100.0	101.0	100.0	105.0
CO_2	30.0	23.0	24.0	24.0	23.0	24.0	23.0	30.0
Anion Gap	9.0	17.0	15.0	18.0	20.0	17.0	19.0	11.0
BUN	7.0	16.0	14.0	15.0	12.0	10.0	13.0	8.0
Creatinine	1.0	1.2	1.0	1.1	1.3	1.0	1.1	1.0
BUN/Creatinine	7.0	13.0	14.0	13.0	9.0	10.0	9.0	8.0
Cholesterol, Total	83.0	106.0	106.0	92.0	95.0	88.0	96.0	86.0
Triglycerides	16.0	28.0	53.0	31.0	51.0	36.0	50.0	30.0
LDL-Cholesterol	35.0	56.0	51.0	41.0	40.0	36.0	40.0	34.0
VLDL-Cholesterol	3.0	5.0	10.0	6.0	10.0	7.0	9.0	6.0
Uric Acid	0.4	0.3	0.2	0.1	0.3	0.1	0.1	0.2
Phosphorus	6.8	6.3	7.4	7.5	8.2	6.9	8.0	6.9
Calcium	9.5	10.0	9.7	9.1	9.9	9.5	9.5	9.4
Total Protein	6.3	7.4	6.9	6.5	6.9	6.5	6.9	6.4
Albumin	3.2	3.7	3.5	3.3	3.5	3.2	3.5	3.3
Globulin	3.1	3.7	3.4	3.2	3.4	3.3	3.7	3.2
A/G Ratio	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Bilirubin, Total	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.2
Bilirubin, Direct	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1
Alkaline Phosphatase	131.0	140.0	131.0	155.0	159.0	138.0	146.0	134.0
GGT	50.0	47.0	50.0	46.0	51.0	49.0	50.0	49.0
AST (SGOT)	27.0	28.0	38.0	40.0	20.0	27.0	29.0	28.0
ALT (SGPT)	36.0	37.0	43.0	39.0	38.0	39.0	39.0	37.0
LDH	467.0	493.0	440.0	451.0	472.0	354.0	384.0	450.0
CPK	670.0	1677.0	2235.0	422.0	313.0	689.0	386.0	464.0
Iron	101.0	131.0	167.0	96.0	41.0	104.0	110.0	105.0

* Light-activated MC540

Table 7. In vivo toxicity of p-MC540* in pigs

Laboratory Test	Baseline	Day 0 2 mg/kg Injection	Day 1	Day 3 3 mg/kg Injection	Day 5	Day 7 5 mg/kg Injection	Day 15	Day 30
CBC w/Differential Platelet Count								
WBC	13.0	16.1	16.7	16.1	22.3	18.7	15.1	14.3
RBC	6.3	7.4	6.9	7.3	6.8	6.1	6.3	6.4
Hemoglobin	11.2	13.3	12.2	11.9	12.0	10.8	11.1	11.2
Hematocrit	33.0	39.6	36.5	37.8	36.5	33.0	34.0	33.0
MCV	53.0	53.0	53.0	54.0	54.0	55.0	54.0	53.0
MCH	17.9	17.9	17.8	17.7	17.8	17.9	17.9	17.9
MCHC	33.9	33.6	33.4	34.1	32.9	32.7	33.9	33.8
RDW	19.6	19.9	19.6	19.7	21.8	21.0	20.0	20.0
Platelet Count	310.0	305.0	275.0	296.0	397.0	273.0	305.0	310.0
MPV	9.2	10.9	10.7	10.8	9.1	8.8	9.4	9.3
Lymphocytes %	66.5	52.0	49.0	54.0	38.0	65.0	66.0	67.0
Monocytes %	8.9	15.0	4.0	10.0	10.0	3.0	9.0	10.0
Granulocytes %	24.6	33.0	47.0	36.0	52.0	32.0	25.0	23.0

* Light-activated MC540

Table 8. In vivo toxicity of p-MC540* in dog

Laboratory Test	Baseline	Day 0 20 mg/kg Injection	Day 1	Day 3	Day 7	Day 15	Day 30
SMAC-28							
Glucose	80.5	86.0	91.0	108.0	96.0	88.0	90.0
Sodium	146.0	144.0	144.0	147.0	142.0	141.0	146.0
Potassium	4.0	4.8	4.7	4.7	4.4	4.8	4.0
Chloride	110.5	105.0	101.0	109.0	107.0	106.0	111.0
CO_2	21.5	21.0	21.0	24.0	22.0	15.0	22.0
Anion Gap	14.0	18.0	22.0	14.0	19.0	18.0	14.0
BUN	15.0	28.0	20.0	16.0	18.0	12.0	15.4
Creatinine	1.3	1.0	1.3	1.0	1.4	1.3	1.3
BUN/Creatinine	11.5	18.0	23.0	16.0	14.0	9.0	11.8
Cholesterol, Total	152.5	174.0	182.0	172.0	169.0	200.0	164.0
Triglycerides	24.0	86.0	88.0	78.0	50.0	30.0	36.0
LDL-Cholesterol	103.0	112.0	120.0	112.0	100.0	149.0	138.0
VLDL-Cholesterol	4.5	17.0	17.0	15.0	20.0	6.0	10.0
Uric Acid	0.35	0.3	0.6	0.3	0.4	0.5	0.4
Phosphorus	4.5	3.9	4.0	4.8	4.9	5.4	4.8
Calcium	10.15	10.1	10.2	10.4	10.2	10.3	10.1
Total Protein	6.0	6.1	6.9	6.2	7.0	8.0	7.6
Albumin	3.7	3.7	4.1	3.7	3.8	4.6	3.8
Globulin	2.35	2.4	2.8	2.5	2.6	3.4	3.8
A/G Ratio	1.57	1.5	1.5	1.5	1.5	1.4	1.0
Bilirubin, Total	0.1	0.2	0.2	0.2	0.2	0.3	0.2
Bilirubin, Direct	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Alkaline Phosphatase	41.0	56.0	78.0	46.0	60.0	70.0	64.0
GGT	5.5	1.0	1.0	3.0	5.4	4.4	4.9
AST (SGOT)	30.0	166.0	68.0	38.0	69.0	560.0	420.0
ALT (SGPT)	33.0	115.0	35.0	82.0	78.0	900.0	160.0
LDH	70.0	108.0	94.0	169.0	149.0	1355.0	154.0
CPK	184.0	2046.0	782.0	139.0	160.0	346.0	189.0
Iron	171.0	215.0	60.0	203.0	79.0	121.0	169.0

* Light-activated MC540

A dog was injected with 20 mg/kg pMC540 through the cephalic vein (single-dose administration) and was followed for a period of 30 days postinjection. SMAC-28 analysis, a CBC with differential, and a coagulation-factor profile were done as in the studies using pigs. The data indicated no abnormality in terms of serum electrolytes, renal functions, complete blood picture, or coagulation profile (Tables 8, 9). Levels of hepatic enzymes, namely, serum glutamic oxaloacetic transaminase (AST) and serum glutamic pyruvic transaminase (ALT) were elevated following drug administration; however, levels of gamma-glutamyl transpeptidase (GGT), a more sensitive indicator

Table 9. In vivo toxicity of p-MC540* in dog

Laboratory Test	Baseline	Day 0 20 mg/kg Injection	Day 1	Day 3	Day 7	Day 15	Day 30
CBC w/Differential Platelet Count							
WBC	9.5	15.3	14.2	9.9	10.2	13.4	10.4
RBC	6.36	7.6	8.3	7.04	7.1	7.3	7.4
Hemoglobin	14.7	17.9	18.4	16.6	15.4	15.8	15.2
Hematocrit	41.3	50.5	52.0	46.8	47.0	49.0	46.4
MCV	65.0	66.0	63.0	67.0	64.0	60.4	63.4
MCH	22.4	23.6	22.2	23.6	24.0	21.8	21.8
MCHC	35.3	35.5	35.3	35.5	34.5	36.2	34.9
RDW	13.3	13.7	12.6	13.7	13.4	13.1	13.6
Platelet Count	124.0	310.0	116.0	261.0	201.0	126.0	124.0
MPV	11.6	8.7	11.5	8.9	10.6	11.4	11.8
Lymphocytes %	30.0	21.0	15.0	20.0	18.0	26.0	30.0
Monocytes %	5.0	3.0	4.0	10.0	9.0	5.0	5.0
Granulocytes %	51.0	71.0	70.0	59.0	59.0	66.0	54.0
Eosinophils %	9.0	4.0	9.0	10.0	9.4	8.0	7.0
PT Profile							
PT	8.3	8.0	8.0	8.0	8.2	8.4	8.3
PTT	205.0	205.0	>205.0	>205.0	>205.0	>205.0	205.0
Fibrinogen	168.0	166.0	164.0	181.0	201.0	200.0	174.0

* Light-activated MC540

of hepatic damage, were within normal limits. Total bilirubin levels were also unchanged following drug injection, suggestive of minimal damage to liver parenchyma.

Discussion

Photoactive compounds have successfully been used for the treatment of certain malignancies, e.g., hematoporphyrin derivative (a multicomponent mixture) is currently undergoing clinical trial for its limited use [6, 7, 18]. We have been interested in the development of antitumor and antiviral agents that do not depend on the delivery of light at the target site such that they can be used as systemic agents. Recent work has resulted in the generation of such light-activated agents in our laboratory by a process termed "preactivation" [3, 12, 13]. One such compound is the light-activated product of the polymethine sulfonic acid dye MC540 (pMC540). MC540 is a preferential binder of certain types of tumor cells and immature blood cells. Many reports have been published on its role as a photosensitizer [8, 10, 11, 21]. pMC540 is toxic to enveloped viruses and certain types of tumor cells, showing minimal toxicity toward normal peripheral mononuclear cells [12, 13]. The biologically active species in pMC540 remains to be identified, but it appears that the MC540 molecule undergoes attack by singlet oxygen, thereby breaking down and forming toxic products [5]. The present study was aimed at evaluating the in vivo toxicity and organ distribution of pMC540 in animal models.

The lethal dose (single i.v. dose) of pMC540 in DBA/2 mice is 240 mg/kg as compared with 40 mg/kg for MC540. Repeat-dose experiments reveal that the mice tolerate a cumulative dose of up to 400 mg/kg without showing any toxic effect. Results based on single-dose and repeat-dose toxicity data clearly indicate that pMC540 shows significantly reduced toxicity ($P < 0.001$) as compared with nonactivated MC540. Toxicity studies were performed on pigs and a dog for a period of 30 days to assess the damage to any organ system caused by pMC540. The administration of pMC540 did not produce signs of immediate hypersensitivity, respiratory distress, tachycardia, or fever in pigs given an accumulated dose of 10 mg/kg or in dogs given a single dose of 20 mg/kg. Pigs receiving incremental doses of pMC540 showed no abnormality in terms of serum electrolytes, renal functions (serum creatinine, <1 mg/dl), liver-function tests, or blood-coagulation profiles. The unusually high levels of CPK observed might have been due to the i.m. injection of animals with 5 mg Valium or 8 mg ketamine to restrain them for blood sampling on days 0 and 1. These levels had returned to the normal range by day 3 and remained so for the duration of the study.

Tests of liver function constitute one of the most important assays in studies involving drug toxicity. Abnormalities in the conjugation of lipid-soluble, unconjugated bilirubin to water-soluble bilirubin glucoronide manifest as an elevation of total bilirubin (normal value, <1.0 mg/dl) in the serum, indicating clinical jaundice. Our data reveal that there was no elevation of serum bilirubin in animals injected with pMC540. Functional and mechanical damage to the liver is assessed by enzyme assays specific for liver functions. Alkaline phosphatase, which hydrolyzes phosphate esters, is strikingly elevated in drug-induced cholelithiasis [1]. Levels of alkaline phosphatase in an animal given 20 mg/kg pMC540 showed a slight increase but were within the normal range, suggesting no obstructive lesion of the biliary tract.

Serum transaminase activity is elevated in hepatocellular damage caused by chemicals or toxins [17]. We deter-

mined the activity of the three commonly assayed aminotransferases from the serum of animals injected with pMC540 for a period of 30 days. AST and ALT levels were elevated in animals injected with 20 mg/kg pMC540, with no clinical sign of hepatotoxicity being observed. These values may reflect nonspecific enzyme induction unrelated to the drug itself. This hypothesis was consolidated by assaying for the most sensitive indicator of hepatic damage, i.e., serum levels of GGT. Elevation of serum levels of GGT (normal range, 5–40 komen units) is observed in minimal hepatocellular damage [16]. In our study, the levels of GGT remained well below the normal limits for the duration of the study, suggesting a lack of damage to the biliary tract. Moreover, the results indicated no damage to the functional ability of the liver to synthesize albumin and globulin. Serum levels of albumin remained unchanged from the baseline readings throughout the duration of the study, again indicating the absence of hepatic damage (serum albumin levels are decreased in hepatocellular damage and there is reactive hyperglobulinemia, resulting in an A/G ratio of <1). Furthermore, in hepatic damage, the ability of the liver to convert ammonia to urea is impaired, resulting in an increase in serum levels of ammonia [15]. Our data show that there was no elevation of serum ammonia levels in animals injected with pMC540 at any point during the study.

The coagulation profiles of injected animals showed a lack of abnormalities. Prothrombin time (PT) was unchanged after drug administration, reflecting normal activities of prothrombin, fibrinogen, and factors V, VII, and X. As all of these factors are synthesized by the liver, PT is an indirect test of normal liver function. Similarly, the partial thromboplastin time (PTT) remained unchanged, indicating normal activities of prothrombin, fibrinogen, and factors V, VII, IX, and XI. These results show that pMC540 does not cause endothelial damage and is nonthrombogenic. The biodistribution data indicate that >70% of the pMC540 was eliminated within 6 h in the urine and feces. The peak plasma level was reached within 30 min of an i.p. injection, and almost half of this was accounted for in the brain (48.8%). A rapid rate of elimination could allow the use of a low repeat-dose regimen with minimal potential for toxicity. The ability of pMC540 to cross the blood-brain barrier may be a desirable property for the intracranial manifestations of systemic viral infections, e.g., human immunodeficiency virus type 1.

The rapid rate of elimination via the kidneys suggests that a water-soluble metabolite is formed on the activation of MC540. Being a hydrophobic dye, MC540 is lipophilic, remains essentially nonionized at physiological pH, and often binds strongly to plasma proteins. Consequently, like most lipophilic, pharmacologically active agents, MC540 would be expected to have a prolonged duration of action. However, it appears that on its photoactivation, a new metabolite is formed that is water-soluble and readily filtered at the glomerular tubules. The biotransformed product(s) appeared to be well tolerated in all species of animals tested. However, it should be noted that because of the different routes of administration, bioavailability, and absorption characteristics in mice (i.p.) and pigs and dogs (i.v.), an interspecies comparison of the data is not possible. The i.v. injection of pMC540 into mice was not possible at the time of the study due to the nonspecific toxicity of the carrier solvent (2.5% ethanol) in some control animals. The probable reason for this toxicity is believed to be the volume (300 µl) of the injection solution containing 2.5% aqueous ethanol. Attempts to reduce the volume of the solvent failed, since pMC540 lost its biologial activity on lyophilization and thus could not be concentrated further. However, other methods for concentrating pMC540 that do not compromise its antitumor and antiviral activities are currently under investigation.

The data presented herein suggest that pMC540 is very well tolerated in vivo. However, it is obvious that no drug is totally innocuous and that some toxicity is bound to occur at higher doses. The present data support further investigations of the in vivo effects of pMC540.

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