J. Toxicol. Pub. Health
Vol. 22, No. 1, pp. 55-59 (2006)

Official Journal of
Korean Society of Toxicology
Available online at http://www.toxmut.or.kr

Toxicity of Hematoporphyrin-Coated Magnetic Ferrofluid in Rats

Youn-Hwan Hwang, Jong-Hwan Lim, Byung-Kwon Park, Myoung-Suk Kim, Chong-Oh Kim[1] and Hyo-In Yun

Division of Veterinary Pharmacology and Toxicology, College of Veterinary Medicine, Chungnam National University, Gung-dong 220, Yuseong-gu, Daejeon
[1]Department of Materials Science and Engineering, Chungnam National University, Gung-dong 220, Yuseong-gu, Daejeon 305-764, Korea

Received December 8, 2005; Accepted January 4, 2006

ABSTRACTS. The purpose of this study was to investigate the toxicity of hematoporphyrin-coated magnetic ferrofluid (HP-MF) through intravenous administration in Sprague-Dawley rats. Each group was treated with either saline, or the HP-MF at 0.5, 1, 1.5, 2 and 4 ml/kg body weight (b.w.) for the observation of survival rate, clinical symptoms, laboratory values and histopathological findings. In this study, HP-MF was evaluated for the survival rates, symptoms, laboratory values and histopathological examination after treatments. The result revealed that the animals in the group of HP-MF at 2 and 4 ml/kg b.w. showed some lethality. In serum biochemistry, the levels of AST, ALT and ALP were increased in the MF and HP-MF treated groups. However, histopathological examination for the suspected organs showed no evidence of hepatotoxicity and nephrotoxicity of typical iron poisoning. Though the toxicity of HP-MF was higher than that of HP, long retention of hematoporphyrin via HP-MF provides additional benefit over conventional hematoporphyrin. HP-MF could be utilized as a potential photodynamic agent in cancer therapy. It is suggested to develop an efficient external magnetic device to attract hematoporphyrin in the target site, thereby enabling to administering a small amount of HP-MF.

Keywords: Magnetic fluid, Hematoporphyrin, Toxicity.

INTRODUCTION

Magnetic targeted carriers (MTCs) system, one form of regional drug targetings, was proposed for the first time by Widder *et al.* (1981) who utilized magnetically responsive materials. It is the concept of magenetic targeting that drugs concentrate in the targeted site using external magnetic field, thereby the actions of drug prolong as increasing the retention of MTCs in target site. Magnetic ferrofluids, which have iron-containing materials with diameters below 15 nm dispersed in a nanogenic matrix, have many possibilities of application in biology, medical diagnosis and therapy (Halbreich *et al.*, 1998; Lacava *et al.*, 2002).

Nanoparticle magnetic ferrofluid coated with hematoporphyrin (Kim *et al.*, 2002) was developed by the Research Center for Advanced Magnetic Materials (Daejeon, Korea). Hematoporphyrin, a photosenitizing drug, is retained with some degree of selectivity in the tumor and activated by light of an adequate wavelength to produce local cytotoxic effects mediated mainly via singlet oxygen (Pahernik *et al.*, 1998; Fuchs *et al.*, 2000). In magnetic ferrofluids, magnetic particles can be coated with a photosensitizer in the condition of colloidal suspension, thereby enabling to guide them in the targeted sites by applying external magnetic fields. However, the safety of the magnetic ferrofluid coated with hematoporphyrin *in vivo* must be addressed before clinical applications.

The purpose of this study was to investigate the toxicity of HP-coated magnetic ferrofluid through intravenous administration in Sprague-Dawley rats.

MATERIALS AND METHODS

Preparation of HP-coated magnetic ferrofluid

The HP-coated magnetic ferrofluid (HP-MF) was obtained from the Research Center for Advanced Magnetic Materials, Chungnam National University (Dae-

Correspondence to: Hyo-In Yun, Division of Veterinary Pharmacology and Toxicology, College of Veterinary Medicine, Chungnam National University, Gung-dong 220, Yuseong-gu, Daejeon, Korea
E-mail: hiyun@cnu.ac.k

jeon, Korea). It was synthesized by coprecipitation from a solution of iron (III) chloride tetrahydrate and iron (II) chloride hexahydrate using ammonium hydroxide. The stabilization of ferrofluid was achieved by coating the particle surface with organic surfactants. The dispersion contained 2.5% particles (25 mg iron oxide in 1 ml magnetic ferrofluid). The particles consisted of Fe_3O_4 and contained 60% pure iron, such that there was a total iron content of roughly 15 mg/ml magnetic ferrofluid. The particle diameter was below 10 nm and the color of magnetic ferrofluid was brown. The concentration of hematoporphyrin in the HP-coated magnetic ferrofluid was 0.3 mg/ml for magnetite particles of the prepared magnetic ferrofluid. The hematoporphyrin was prepared in the isotonic saline at a concentration of 0.3 mg/ml.

Animals

Male Sprague-Dawley rats, weighing between 190 and 220 g at the age of 6~7 weeks, were used for these studies. They were obtained from Samtaco (Anyang, Korea) and acclimated for one week before experiments. Only healthy animals were used in these studies. The animals were supplied with laboratory animal feed pellet (Samyang, Korea) and filtered water *ad libitum*. Animal environmental control unit for rats (GOG Environmental Control Unit, MJ, Korea) was run at temperature 23 ± 2°C, humidity 55 ± 3%. Lighting was controlled to give a 12-hrs light-dark cycle during the acclimation period, and all animals were reared in the dark room during the experiment period.

Survivals and behavior

Twenty-five rats were entered into 5 groups of each 5 rats for the survival study, and all animals of each group were treated by the intravenous administration of either saline at 4 ml/kg body weight (b.w.), or HP-MF at 0.5, 1, 2 and 4 ml/kg b.w. through the tail vein. Death and clinical symptoms of animals were observed for 4 weeks after administration.

Another 20 rats were randomly divided into 4 groups of each 5 rats for the weight, behavior and laboratory studies. All animals were treated with each drug (1.5 ml/kg b.w.) through the tail vein as follows. Group 1 (Saline) received isotonic saline solution; Group 2 (HP) received the hematoporphyrin (0.3 mg/ml); Group 3 (HP-MF) received the magnetic ferrofluid coated with hematoporphyrin; and Group 4 (MF) received the magnetic ferrofluid.

All drugs were injected over 1 min into the tail vein. Blood samples to determine hematological and serum chemical values were taken prior to and 1, 3, 7, and 14 days after treatment. In addition, animal behavioral changes and weights were observed at the same time.

Blood analysis

Blood samples were either collected into EDTA-added bottles for hematological tests or into no anticoagulant bottles for the blood chemistry. HEMAVET (CDC, USA) was used for the analysis of hematological parameters. ALT (alanine amionotransferase), AST (aspartate aminotransferase), ALP (alkaline phosphatase), blood urea nitrogen (BUN) and creatinine values were measured by VetTest 8008 (IDEXX Lab. Inc., USA).

Histopathological examination

Other 12 rats were divided into 2 groups, and animals of each group received HP-MF or HP (0.3 mg/ml) at 1.5 ml/kg through the tail vein. Two hours and 7 days after administration, three animals of each group were sacrificed by ethyl ether, and relevant organs were histologically examined with the standard procedures for H&E or Perl's stain for the determination of iron.

A Confocal Microscope Leica-Mirbe, software Leica TSCNT 1.5.451, equipped with Kripton-Argon laser was used for the detection of hematoporphyrin in tissues. For the detection of cell-bound HP, 585 nm long-pass filter was used in channel 1 (red), while 505~550 nm band-pass filter was used in channel 2 (green) for background.

Statistics

In general, data were reported as means ± SD and statistical analyses were performed using a computer software program (Microsoft Excel, Microsoft, USA). The significant correlation between the time and group was determined by a two-way analysis of variance (ANOVA). The differences between data of groups were performed using different variance, two-tailed *t*-tests. The difference between the data of groups was considered significant at the level of $P < 0.05$.

RESULTS AND DISCUSSION

The objective of these studies was to assess the toxicity of a newly developed HP-MF in SD rats. The toxicity testing is able to indicate harmful effects and to explore the mechanism of each toxic action, thereby enabling to avoid, or at least to minimize or treat them.

The body weight changes of all groups treated with different volumes of HP-MF were not statistically different as compared to those of the control group (data not shown). The clinical symptoms showed volume-dependency with the greatest symptoms observed in rats treated with HP-MF at 4 ml/kg b.w. (data not shown).

Rats in the high dose group of HP-MF (2 ml/kg b.w. and 4 ml/kg b.w.) revealed diarrhea, tachycardia, tachypnea, seizure, crawing position, clonic convulsion and shock immediately after the treatment, but these symptoms were not shown 1 day after treatment in survival animals. Groups treated with smaller volumes of HP-MF showed depression, mild seizures and anorexia, but these symptoms disappeared 1 day after the treatment. Any dead animal in groups treated MF with 0.5 and 1 ml/kg b.w. was not observed. All animals in the group of 4 ml/kg b.w. died within 2 hrs after treatment (Fig. 1).

Mechanical occlusion with ferrofluid in magnetic drug targeting was reported in high concentration of ferrofluid (Gielbel *et al.*, 1985). The actual volume of ferrofluid as a vehicle to concentrate drugs locally in tumors was less than 0.5 ml/kg b.w., equivalent to 3 mg/kg as iron for actual treatment (Lübbe *et al.*, 1999). In the present study, the medium lethal dose of magnetic ferrofluid after venous administration in rat was 2.7 ml/kg b.w. equivalent to 40 mg/kg as iron. These results implied that the HP-MF could be safe in the viewpoint of the mechanical obstruction of microcirculation. Moreover, HP-MF has several advantages in the photodynamic therapy: magnetic field-dependent localization of hema- toporphyrin, more selective attack to the target site, effect of reactive oxygen radical induced by external magnetic fields, etc.

On the while, Kuzensove *et al.* (1999) reported that the LD_{50} of dextran-coated magnetite nanospheres was 5 g/kg for mice, 1 g/kg for rabbits and 0.7 g/kg for dogs after intravenous application. Dextran-coated magnetite

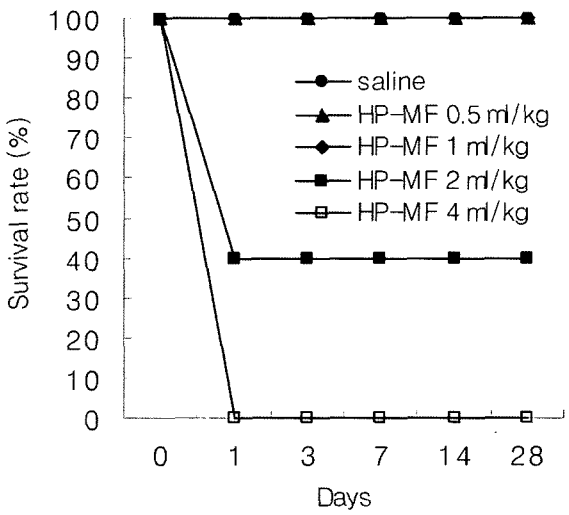

Fig. 1. Survival rates of rats for 4 weeks postinjection with various volumes.

was safer than aqueous magnetic ferrofluid in this study. These results were arisen from different preparation procedures between ferromagnetic iron dextran particles and HP-MF. Ferromagnetic iron dextran particles were prepared by reacting with a mixture of ferrous chloride and ferric chloride with dextran polymers under alkaline conditions. However, HP-MF in the present study were prepared by reacting a mixture of ferrous chloride and ferric chloride with decanoic acid as the first surfactant, nanonoic acid as the second surfactant, heamatoprophyrin, organic acid as the solvent of surfactant under alkaline conditions.

Table 1. Serum biochemistry after intravenous injection of different magnetic ferrofluids at 1.5 ml/kg body weight

Time after treatment	Groups[a]	Serum biochemical parameter[b] (Mean ± SD)				
		ALT (IU/l)	AST (IU/l)	ALP (IU/l)	BUN (mg/dl)	Creatinine (mg/dl)
Day 1	Saline	55.7 ± 4.7	73.0 ± 11.1	270.0 ± 17.7	18.0 ± 2.1	0.3 ± 0.0
	HP	56.7 ± 0.6	86.0 ± 17.4	268.3 ± 29.2	18.1 ± 1.5	0.3 ± 0.0
	HP-MF	80.0 ± 12.3*	119.7 ± 13.2*	314.7 ± 93.6	19.6 ± 2.3	0.3 ± 0.0
	MF	82.0 ± 10.6*	114.7 ± 10.1*	439.7 ± 33.7*	21.8 ± 5.7	0.3 ± 0.0
Day 3	Saline	55.6 ± 6.7	67.0 ± 9.5	266.3 ± 22.0	18.8 ± 1.5	0.3 ± 0.0
	HP	52.7 ± 9.1	70.0 ± 21.9	253.7 ± 23.0	18.2 ± 1.8	0.3 ± 0.0
	HP-MF	69.7 ± 6.1*	121.0 ± 16.0*	337.3 ± 54.3	19.6 ± 0.6	0.3 ± 0.0
	MF	79.3 ± 6.4*	150.7 ± 10.7*	350.0 ± 37.4*	19.9 ± 5.2	0.3 ± 0.1
Day 7	Saline	54.7 ± 7.1	64.3 ± 13.1	266.7 ± 20.6	16.4 ± 1.1	0.3 ± 0.0
	HP	58.0 ± 12.1	78.0 ± 13.5	268.3 ± 27.2	18.2 ± 2.4	0.3 ± 0.1
	HP-MF	70.0 ± 13.1*	121.7 ± 15.7*	328.0±28.6*	16.5 ± 2.7	0.3 ± 0.1
	MF	76.7 ± 6.8*	135.7 ± 20.6*	331.7 ± 13.6*	18.8 ± 2.5	0.3 ± 0.1
Day 14	Saline	56.3 ± 4.0	66.7 ± 11.2	266.0 ± 13.7	16.0 ± 1.6	0.3 ± 0.1
	HP	55.0 ± 7.5	65.7 ± 16.8	268.0 ± 19.7	15.7 ± 3.7	0.3 ± 0.1
	HP-MF	72.0 ± 7.5*	117.7 ± 21.0*	318.3 ± 30.2*	16.5 ± 2.1	0.3 ± 0.1
	MF	76.3 ± 6.1*	141.3 ± 20.8*	317.3 ± 20.6*	20.1 ± 3.2	0.3 ± 0.0

[a]HP, hematoporphyrin (0.3 mg/ml); HPMF, HP-coated magnetic ferrofluid; MF, magnetic ferrofluid.
[b]ALT, alanine amionotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; BUN, blood urea nitrogen.
*Statistically different from saline at $P < 0.05$.

Large magnetic microspheres can physically irritate the surrounding tissues or even embolize small blood vessels and capillaries. In the present study, magnetic ferrofluids with diameters below 15 nm were observed to show less physical toxicity such as embolism. However, toxic effects after the administration of various magnetic ferrofluids were dependent on doses. Therefore, the toxic effects could be caused by soluble factors which might include surfactants and organic solvents from the magnetic ferrofluids, or breakdown products during storage.

Hematological parameters did not change the baseline after the injection of different magnetic ferrofluids (data not shown). The serum biochemical levels of each group were shown in Table 1. ALT, AST and ALP levels of different magnetic ferrofluid treatment groups

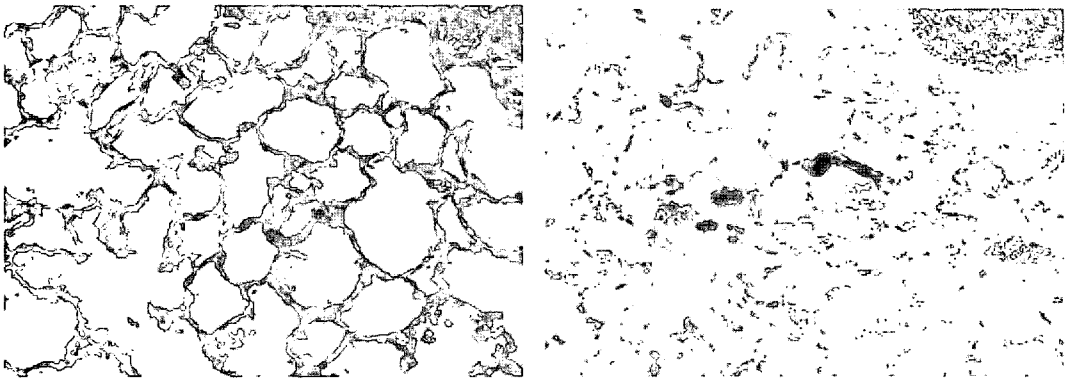

Fig. 2. Sections of lung stained with H-E (A) and Prussian blue (B) 2 hrs after treatment with HP-MF at 1.5 ml/ kg body-weight. Magnification ×400.

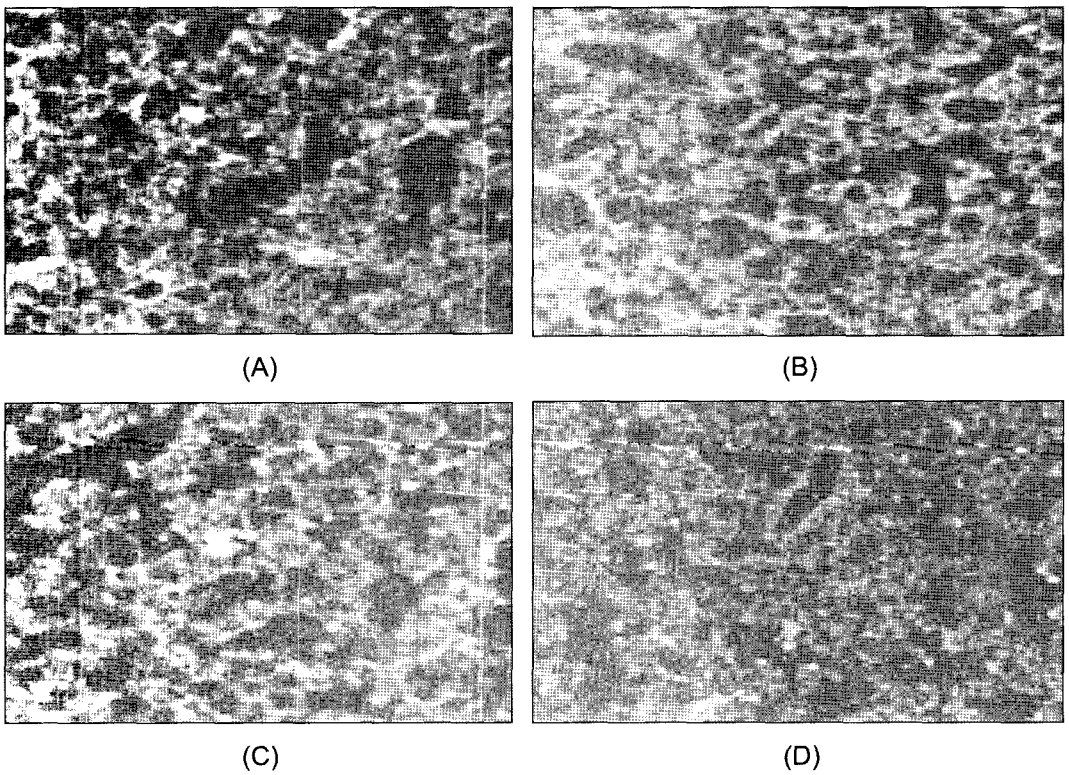

Fig. 3. Confocal laser microscopic observation of hematopophyrin in lung at 2 hrs and 7 days after treatment with 1.5 ml/kg body weight. (A) hematoporphyrin (0.3 mg/ml), 2 hrs after treatment; (B) hematoporphyrin (0.3 mg/ml), 7 days after treatment; (C) HP-MF, 2 hrs after treatment; (D) HP-MF, 7 days after treatment.

increased as compared with the saline group during the all experimental periods ($P < 0.05$).

In the acute iron poisoning, the elevation of ALT and AST values arose due to some sort of hepatic degeneration (Osweiler, 1996). Hepatotoxicity was found to occur at as low as 1,700 μg/dl of human serum iron concentration (Tenenbein, 2001). When iron exceeds the needs of body, it produces reactive oxygen radical species such as superoxide anion and hydrogen peroxide (Cairo *et al.*, 2002). We assumed that the changes on the serum biochemistry shown in our experiment may result from the presence of iron particles of magnetic ferrofluid in liver tissues or from the added organic acid and surfactant during the ferrofluid synthesis.

In contrast with the blood chemistry, there was no evidence of tissue damages. The lack of toxic effects can be accounted for by considering the *in vivo* effects from the magnetic ferrofluids such as local inflammation, leukocyte infiltration and severe diffuse hepatocellular necrosis of the liver as a typical iron poisoning lesion. Phagocytized iron particle by Kuffer cells and alveolar macrophages were shown in the treated groups of HP-MF (Fig. 2). Uptake and successive elimination of the iron particles by the reticuloendothelial system were confirmed by the histological data.

The distribution sites of hematoporphyrin were found to emit the flourescence. The site and fluorescence intensity were observed to be similar between HP and HP-MF groups on the confocal microscopy (Fig. 3). However, HP-MF was longer retained than hematoporphyrin. In other words, HP-MF is regarded to have longer action than that of conventional photodynamic therapy in the target site.

CONCLUSIONS

Although HP-MF showed some toxic effects in the group treated with larger volume, histopathlogical examination of suspected organs showed no evidence of iron poisoning which accompanies hepatotoxicity and nephrotoxicity. Hematoporphyrin from the HP-MF showed long retention, providing additional benefits over conventional hematoporphyrin. HP-MF could be utilized as a potential photodynamic agent against cancer. It is, therefore, suggested to develop an appropriate external magnetic device enabling to attract hematoporphyrin in the target site, enough to administer a small amount of HP-coated magnetic ferrofluid. Physiological as well as pharmacological parameters in HP-coated magnetic ferrofluids warrant further investigation. This is because the efficacy of *in vivo* drug targeting with ferrofluids critically depends on physiological parameters.

ACKNOWLEDGEMENTS

This study was supported by a grant of the International Mobile Telecommunications 2000 R&D Project, Ministry of Information & Communication, Republic of Korea.

REFERENCES

Cairo, G., Recalcati, S., Pietrangelo, A. and Minotti, G. (2002): The iron regulatory proteins: targets and modulators of free radical reactions and oxidative damage. *Free. Radic. Biol. Med.*, **32**, 1237-1243.

Fuchs, J., Weber, S. and Kaufmann, R. (2000): Genotoxic potential of porphyrin type photosensitizers with particular emphasis on 5-aminolevulinic acid: implications for clinical photodynamic therapy. *Free. Radic. Biol. Med.*, **28**, 537-548.

Giebel, W., Wagner, H. and Scheibe, F. (1985): Preliminary electrophysiological data after the obliteration of cochlear blood vessels by the action of a magnetic field on circulating iron particles. *Arch. Otorhinolaryngol.*, **242**, 337-341.

Halbreich, A., Roger, J., Pons, J.N., Geldwerth, D., Silva, M.F.D., Roudier, M. and Bacri, J.C. (1998): Biomedical applications of maghemite ferrofluid. *Biochimie*, **80**, 379-390.

Kim, J.H., Kwon, S.K. and Kim, C.O. (2002): Adhesion of photosensitizer to ferrofluids for use in photodynamic therapy. *Nanotechnology*, **13**, 610-614.

Kuzensove, O.A., Brusentsov, N.A., Kuznetsov, A.A., Yurchenko, N.Y., Osipov, N.E. and Bayburtskiy, F.S. (1999): Correlation of the coagulation rates and toxicity of biocompatible ferromagnetic microparticles. *J. Magn. Magn. Mater.*, **194**, 83-89.

Lacava, L.M., Lacava, Z.G.M., Azevedo, R.B., Chaves, S.B., Garcia, V.A.P., Silva, O., Pelegrini, F., Buske, N., Gansau, C., Silva, M.F.D. and Morais, P.C. (2002): Use of magnetic resonance to study biodistribution of dextran-coated magnetic fluid intravenously administered in mice. *J. Magn. Magn. Mater.*, **252**, 367-369.

Lübbe, A.S., Bergemann, C., Brock, J. and McClure, D.G. (1999): Physiological aspects in magnetic drug-targeting. *J. Magn. Magn. Mater.*, **194**, 149-155.

Osweiler, G.D. (1996): Metals and minerals, Toxicology, Lippincott Williams & Wilkins, PA, pp. 179-212.

Pahernik, S.A., Dellian, M., Berr, F., Tannapfel, A., Wittekind, C. and Goetz, A.E. (1998): Distribution and pharmacokinetics of Photofrin in human bile duct cancer. *J. Photochem. Photobiol. B.*, **47**, 58-62.

Tenenbein, M. (2001): Hepatotoxicity in acute iron poisoning. *J. Toxicol. Clin. Toxicol.*, **39**, 721-726.

Widder, K.J., Morris, R.M., Poore, G., Howard, D.P. Jr. and Senyei, A.E. (1981): Tumor remission in Yoshida sarcoma-bearing rats by selective targeting of magnetic albumin microspheres containing doxorubicin. *Proc. Natl. Acad. Sci. USA*, **78**, 579-581.