Role of the Pharmaceutical Excipients in the Tamoxifen Activity on MCF-7 and Vero Cell Cultures

TIZIANA ROSSI[1], VALENTINA IANNUCCELLI[2], GILBERTO COPPI[2], ELISA BRUNI[1] and GIOSUÉ BAGGIO[1]

University of Modena and Reggio Emilia,
[1]Department of Biomedical Sciences, Section of Pharmacology, Via G. Campi 287, and
[2]Department of Pharmaceutical Sciences, Via G. Campi 183, Modena, Italy

Abstract. *Background: Microparticles are used for controlled drug delivery. With the aim of improving both bioavailability and tamoxifen selective toxicity, the activity of tamoxifen embedded in calcium alginate/chitosan microparticles was studied. Materials and Methods: Tamoxifen alone and embedded in microparticles prepared with sodium alginate from Kelco (62% mannuronic acid and 38% guluronic acid) and from Fluka (30% mannuronic acid and 70% guluronic acid) was added to MCF-7 and Vero cultures and evaluated for antiproliferative activity by the MTT test. Results: The use of Kelco or Fluka alginate resulted in different LD_{50} values on Vero and MCF-7 cultures, showing a higher cytotoxicity toward Vero cells treated with tamoxifen embedded in Kelco microparticles (25 μM vs. 48 μM on MCF-7 cells) but a selective toxicity with Fluka microparticles (25 μM and 10 μM on Vero and MCF-7 cells respectively). Conclusion: Microparticle formulation may improve selective toxicity according to the alginate employed: differences in the chemical alginate composition can dramatically change both drug activity and toxicity.*

Tamoxifen is a synthetic antiestrogen that has been used since the 1970s to treat advanced and early-stage breast cancer (1-3). In fact, oral administration of tamoxifen is the treatment of choice for metastatic oestrogen receptor-positive breast cancer. Tamoxifen shows a fairly good oral bioavailability combined with large inter-individual variations and side-effects. With the aim of improving tamoxifen bioavailability, the *in vitro* activity of tamoxifen embedded in calcium alginate/chitosan microparticles was studied. The use of microparticles as drug delivery systems have become important in experimental pharmaceutics and clinical medicine. The particles have been shown to enhance the delivery of certain drugs across a number of natural and artificial membranes and to improve the bioavailability of some drugs (4-7). However, pharmacosurveillance systems point out the role of chemical excipients in the biological activity of drugs in that their link to the active compound can modify the therapeutic response or induce allergic reaction (8-11). We therefore investigated the *in vitro* activity of tamoxifen alone and embedded in microparticles prepared with two different alginates on human breast carcinoma and healthy cell cultures. Finally, 50% lethal dose (LD_{50}) values obtained from the experiments were compared to verify if the microparticle formulation gave a better drug bioavailability (6) and a selective toxicity toward cancer cells.

Correspondence to: Professor Tiziana Rossi, Department of Biomedical Sciences, Section of Pharmacology, University of Modena and Reggio Emilia, Via G. Campi 287, 41100 Modena Italy. Tel: +39 0592055366, Fax: +39 0592055376, e-mail: tiziana.rossi@unimore.it

Key Words: Tamoxifen, MCF-7, bioavailability, toxicity, alginate/chitosan microparticles.

Materials and Methods

Compounds. Tamoxifen: (Z)-2-[4-(1,2-diphenyl-1-butenyl)phenoxy]-*N,N* dimethylethylamine citrate (TAM) kindly donated by Solmag (Mulazzano, Lodi, Italy). Sodium alginate (NaA) extracted from *Laminaria hyperborea* containing 30% mannuronic acid and 70% guluronic acid (low M/G ratio: NaA-L) purchased from Fluka Chemie (Buchs, Switzerland) and sodium alginate extracted from *L. digitata* containing 62% mannuronic acid and 38% guluronic acid (high M/G ratio: NaA-H) purchased from Kelco International (Bagnolex Cedex, France) were used to prepare calcium alginate/chitosan microparticles. Figure 1 shows the general molecular structure of alginates: linear unbranched polymers containing β-(1→4)-linked D-mannuronic acid (M) and α-(1→4)-linked L-guluronic acid (G) residues.

Microparticle preparation. The microparticles, sized less than 3 μm, were prepared by two steps, using both alginates. *Spray-drying step:* TAM methanol solution (0.1%, w/v) was added to both (high and low M/G ratio) aqueous alginate solutions (1%, w/v) and the mixture (10:1 NaA/TAM ratio) was spray dried by a Buechi 190 mini-spray dryer (Buechi Laboratorium, Technik AG, Flawil, Switzerland) to obtain uncrosslinked microparticles. The microparticle TAM concentration was determined as described elsewhere (12).

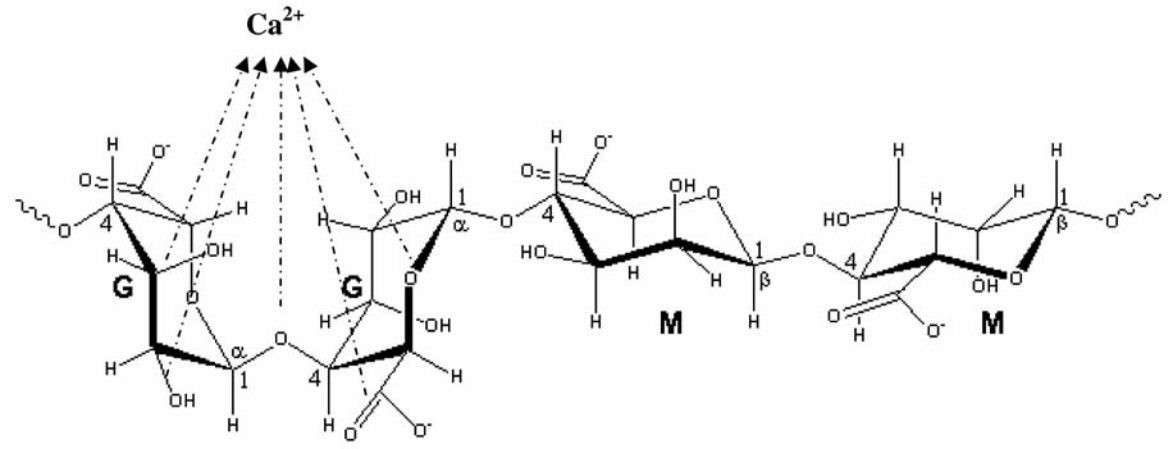

Figure 1. *Alginate molecular structure showing the alternating blocks of 1-4 linked L-guluronic (G) and D-mannuronic (M) acid residues. The figure demonstrates the higher selectivity of guluronic blocks for Ca^{2+} ions (arrows).*

Crosslinking step: Uncrosslinked microparticles were suspended in 10% (w/v) $CaCl_2$ aqueous solution, at a ratio of 0.1/1 w/w.

Cells and cell cultures. MCF-7 cells from human breast carcinoma and Vero cells from African green monkey kidney epithelial cells as control line were used. Cells were cultured in Eagle's minimum essential medium (EMEM) (Lonza, Milan, Italy) enriched with 10% foetal bovine serum (FBS; Lonza) and 1% antibiotic solution (penicillin 50 U/ml and streptomycin 0.5 mg/ml) and 1% L-glutamine in sterile conditions and maintained at 37˚C in a fully humidified atmosphere of 5% CO_2 in air. Once cells were grown to confluence (around 70%), they were transferred together with the culture medium into disposable sterile plates with 94 wells. Living cells were counted by the trypan blue exclusion test to assure an initial inoculum of 35×10^4 cells. The plates were then incubated for 24 h under the same conditions and subsequently the programmed tests were performed.

Cytotoxicity test. After the above incubation time, TAM was dissolved in sterile medium, sterilised by filtration and added to the wells at increasing concentrations (from 10 μM to 150 μM). After a further incubation period (24 h), the methyl thiazole tetrazolium test (MTT) was performed according to the method described by Mosmann (13) to assess cell viability. The MTT test was performed in triplicate on 24, 48 and 72 h cell cultures incubated with the drug alone, the drug embedded in microparticles, and with microparticles without drug (control wells). The results were expressed as percentage of cell growth with respect to the controls.

Statistical analysis. The comparison of data was carried out using the Bonferroni test. The results were considered statistically significant when $p<0.05$.

Results

Effect of TAM and sodium alginate on MCF-7 and Vero cell growth. We first determined the LD_{50} for TAM on MCF-7 and Vero cells. In the present study, significant results were obtained only from 48- and 72-h cultures (Figures 2 a, b) and LD_{50} values (10 μM on Vero and 25 μM on MCF-7 cells) agree with literature data (15-18). As regards the alginate, our data confirm the stimulating effect on cell growth observed by other authors (19). Our study was performed using microparticles prepared with a very low alginate percentage (83% w/v) in order to avoid the uncontrolled proliferation both of Vero and of MCF-7 cells. However, even at the lowest alginate percentage used, both the alginates showed a stimulating dose-related effect on MCF-7 cell growth (Figure 3 a) while such an effect on Vero cells was only apparent at high concentrations (Figure 3 b). Results from 24 h are not shown.

Effect of TAM embedded in microparticles on MCF-7 and Vero cell growth. Literature data suggest that microparticle degradation and consequent drug delivery begin after 24 h (14). In a preliminary study, we demonstrated that the *in vitro* release of TAM from microparticles depends on the alginate: TAM release from NaA-H microparticles showed a three-phase profile, with a first burst phase in which about 40% TAM is delivered, a second fast phase leaching until release of 80% drug, followed by a third sustained phase. TAM release from NaA-L microparticles provided an initial burst phase in which about 40% drug was released in 15 min followed by a second sustained phase (12). The results of the present study confirm these profiles. Histograms of Figures 4 and 5 show the results from the MTT test performed on cell cultures treated with increasing concentrations of the drug embedded in NaA-L and NaA-H microparticles. When TAM was embedded in NaA-H microparticles (Kelco formulation), the LD_{50} increased to 25 μM on Vero cultures and 48 μM on MCF-7 cells. On the contrary, with the Fluka formulation (NaA-L microparticles), the LD_{50} for tumour cells (MCF-7) was significantly reduced thus showing a stronger activity of this formulation on these cells; for Vero cultures the LD_{50} value was no different from that using NaA-H (Table I).

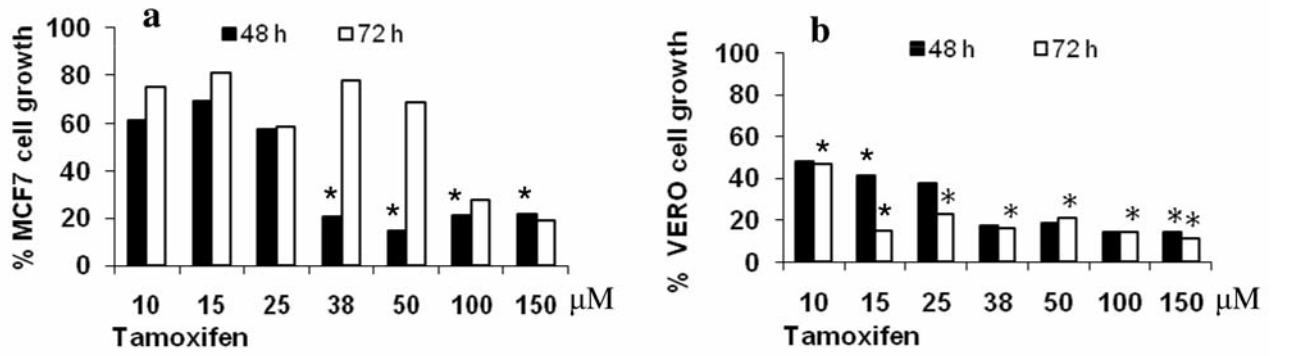

Figure 2. *Antiproliferative activity of increasing tamoxifen concentrations on a) MCF-7 and b) Vero cell cultures as evaluated by the MTT test after 48 h and 72 h of treatment. Data are the mean values from three independent experiments and are expressed as % cell growth with respect to the untreated cultures (control). Significantly different with respect to control (100% growth): *p<0.05 by Bonferroni test.*

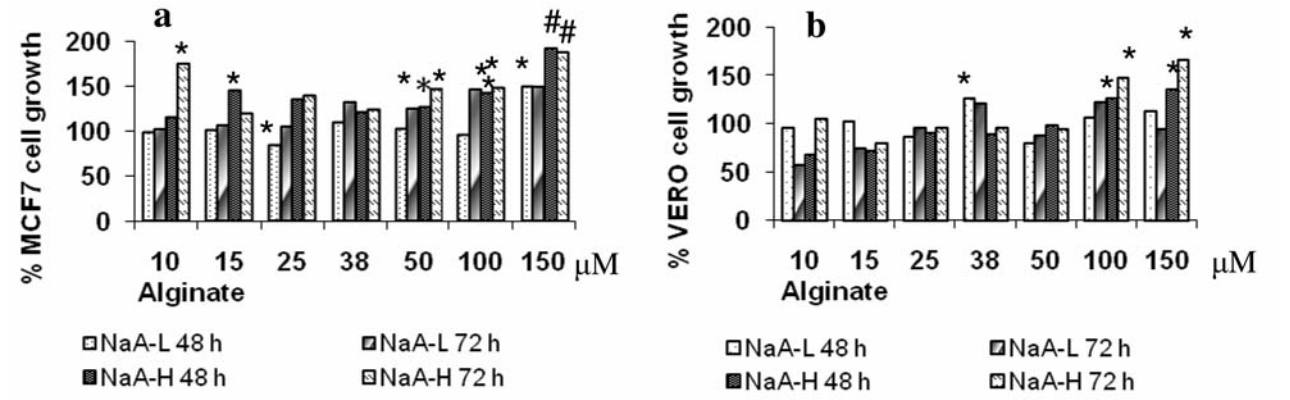

Figure 3. *Effect of increasing concentrations of alginate with low mannuronic/guluronic ratio (NaA-L) and high mannuronic/guluronic ratio (NaA-H) on a) MCF-7 and b) Vero cell cultures as evaluated by the MTT test after 48 h and 72 h of treatment. Data are the mean values from three independent experiments and are expressed as % cell growth with respect to the untreated cultures (control). Significantly different with respect to control (100% growth): *p<0.05 and #p<0.01 by Bonferroni test.*

Discussion

Literature data and clinical experience show that TAM has a fairly good oral bioavailability which, combined with extensive liver metabolism, leads to high therapeutic dosage with the consequence of a higher risk of side-effects (20). The aim of our previous study (12) was to increase TAM oral biovailability or to achieve the desired dose at the tumour site through the use of a microparticulate carrier. In the present study, the use of microparticles as a drug delivery system was shown to modify drug bioavailability. However, our data reveal significant differences in TAM cytotoxicity depending on the chemical composition of the sodium alginate used in the microparticle preparation. Alginate microparticles loaded with TAM were developed by spray-drying technique and cross-linking by calcium ions and chitosan. As previously described, alginate contains mannuronic acid and guluronic acid at a low M/G ratio (Fluka), or a high M/G ratio (Kelco) and the physical and chemical properties of these polymers have been extensively reviewed in several publications (21, 22). TAM would preferably link to guluronic acid, but the higher selectivity for calcium ions prevents it from doing so (Figure 1), therefore, TAM can only be linked by mannuronic acid. When microparticles are prepared with Fluka alginate, the low M/G ratio allows a higher free drug concentration and consequently a higher drug diffusion when microparticles begin to dissolve (23).

In the present study, we observed that TAM embedded in NaA-L microparticles was more active towards MCF-7 cells, showing a selective toxicity towards tumour cells as compared to the control line. The significant reduction of LD_{50} with respect to both TAM alone and TAM embedded in

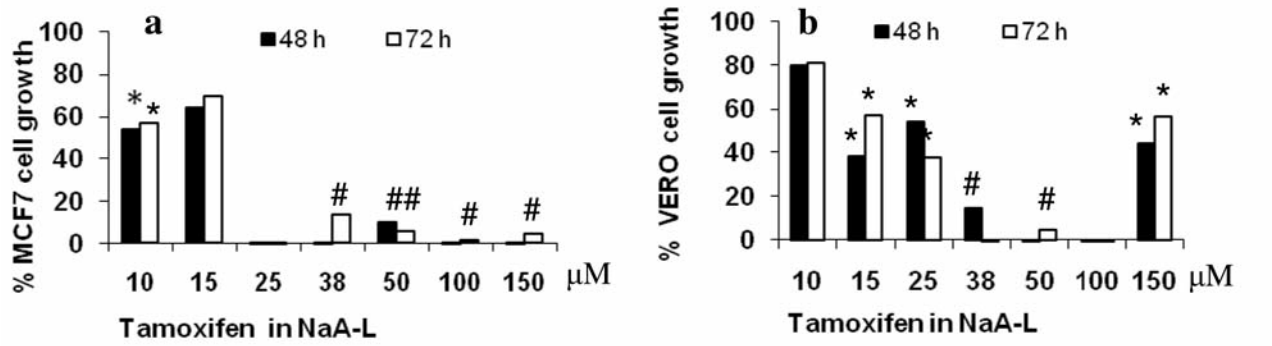

Figure 4. *Antiproliferative activity of increasing concentrations of tamoxifen embedded in NaA-L microparticles on a) MCF-7 and b) Vero cell cultures evaluated by the MTT test after 48 h and 72 h of treatment. Data are the mean values from three independent experiments and are expressed as % cell growth with respect to the untreated cultures (control). Significantly different with respect to control (100% growth): *p<0.05 and #p<0.01 by Bonferroni test.*

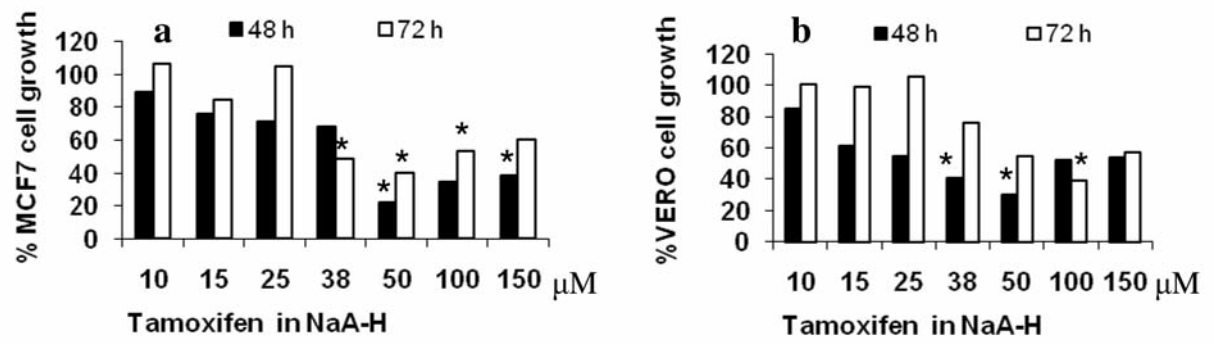

Figure 5. *Antiproliferative activity of increasing concentrations of tamoxifen embedded in NaA-H microparticles on a) MCF-7 and b) Vero cell cultures as evaluated by the MTT test after 48 h and 72 h of treatment. Data are the mean values from three independent experiments and are expressed as % cell growth with respect to the untreated cultures (control). Significantly different with respect to control (100% growth): *p<0.05 by Bonferroni test.*

NaA-H microparticles confirmed the NaA-L formulation as the best drug delivery system for TAM. Only in this case was the drug bioavailability improved, enabling drug toxicity to be reduced and lower dosages employed to gain a selective toxicity toward tumor cells.

The final consideration of the study deals with therapy safety, which can be dramatically changed not only by improving drug bioavailability by means of a new drug delivery system, but even by the choice of the excipients used, which can result in an entirely different toxicological and pharmacological profile of the drug. As pharmacosurveillance agencies report, the substitution of an excipient in the drug formulation does not always guarantee equivalent activity and the therapy must be accurately monitored.

Finally, when human treatment begins with a drug, changing it with a generic one is absolutely discouraged, even if it is defined as being identical or bioequivalent to the brand name drug in dosage form, safety, strength, route of administration and quality. Generics use the same active ingredients but not necessarily the same excipients, and the latter are not always inactive and may link the active molecule thus modifying its bioavailability, activity and toxicity.

Table I. *LD_{50} values for Fluka and Kelco sodium alginate, tamoxifen alone and tamoxifen embedded in alginate microparticles on MCF-7 and Vero cell cultures.*

	LD_{50} (μM)	
Substance	Vero	MCF-7
Na Alginate–L (Fluka)	Not determinable	Not determinable
Na Alginate–H (Kelco)	Not determinable	Not determinable
Tamoxifen	10	25
Tamoxifen in Na Alginate–L	25	10
Tamoxifen in Na Alginate–H	25	48

H, High and L, low mannuronic/guluronic acid ratio.

The U.S. Food and Drug Administration requires that all drugs be safe and effective; the European Agency for the Evaluation of Medicinal Products accepts as bioequivalent all those drugs whose plasmatic concentrations have a variation of ±20% of the original drug, but we conclude that this range is too high in the case of the treatment of severe pathologies as heart, neuronal and tumor diseases.

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