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Co-Encapsulation of Anti-*BCR-ABL* siRNA and Imatinib Mesylate in Transferrin Receptor-Targeted Sterically Stabilized Liposomes for Chronic Myeloid Leukemia Treatment

Liliana S. Mendonça,[1,2] João N. Moreira,[1,2] Maria C. Pedroso de Lima,[2,3] Sérgio Simões[1,2]

[1]Faculty of Pharmacy, Laboratory of Pharmaceutical Technology, University of Coimbra, Pólo das Ciências da Saúde, Azinhaga de Santa Comba, 3000-548 Coimbra, Portugal; telephone: 351-239859950; fax: 351-239827126; e-mail: ssimoes@ci.uc.pt

[2]Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal

[3]Faculty of Science and Technology, Department of Life Sciences, University of Coimbra, Coimbra, Portugal

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ABSTRACT: Chronic myeloid leukemia (CML) is triggered by the *BCR-ABL* oncogene. Imatinib is the first-line treatment of CML; however imatinib resistance and intolerance have been detected in many patients. Therefore, new therapeutic approaches are required. The present work aimed at the development and application of transferrin receptor (TrfR) targeted liposomes co-encapsulating anti-*BCR-ABL* siRNA and imatinib at different molar ratios. The encapsulation yields and drug loading of each molecule was evaluated. Anti-leukemia activity of the developed formulations co-encapsulating siRNA and imatinib and of the combination of Trf-liposomes carrying siRNA and free imatinib under two different treatment schedules of pre-sensitization was assessed. The results obtained demonstrate that the presence of imatinib significantly decreases the encapsulation yields of siRNA, whereas imatinib encapsulation yields are increased by the presence of siRNA. Cytotoxicity assays demonstrate that the formulations co-encapsulating siRNA and imatinib promote a 3.84-fold reduction on the imatinib IC_{50} (from 3.49 to 0.91 μM), whereas a 8.71-fold reduction was observed for the pre-sensitization protocols (from 42.7 to 4.9 nM). It was also observed that the formulations with higher siRNA to imatinib molar ratios promote higher cell toxicity. Thus, the present work describes a novel triple targeting strategy with one single system: cellular targeting (through the targeting ligand, transferrin) and molecular targeting at the *BCR-ABL* mRNA and Bcr-Abl protein level.

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KEYWORDS: triple-targeting; transferrin; anti-*BCR-ABL* siRNA; imatinib; liposomes; CML

Correspondence to: Sérgio Simões

Introduction

Chronic myeloid leukemia (CML) is a malignant myeloid disease triggered by the *BCR-ABL* oncogene, which encodes the constitutively active tyrosine kinase Bcr-Abl protein, this being responsible for the malignant transformation in the disease (Goldman, 2004; Pasternak et al., 1998; Savona and Talpaz, 2008).

Imatinib mesylate revolutionized the treatment of CML, because this drug effectively blocks Bcr-Abl activity and its oncogenic activity (Savage and Antman, 2002; Walz and Sattler, 2006). However, either imatinib or the second generation inhibitors of Bcr-Abl (dasatinib and nilotinib) are not able to overcome all kinds of drug resistance and to completely eradicate the disease (Deininger, 2007; Shah, 2005).

Therefore, the combination of different strategies directed to Bcr-Abl oncoprotein emerges as a therapeutically promising approach to overcome resistance phenomena. The rationale to the use of drug combinations for cancer treatment is based on the heterogeneity of tumor cells and consequently on differences in tumor cell responses to individual drugs (Saxon et al., 1999; Zoli et al., 2001). One significant approach of combined strategies to achieve a more effective treatment for drug-sensitive or drug-resistant leukemia cells is to combine imatinib with gene silencing tools, such as siRNA targeting the oncogene *BCR-ABL*. However, the antitumor activity of drug combinations can be significantly dependent on the molar ratio of the combined drugs. In fact, for the same drug combination some ratios can be synergistic, whereas other ratios can be additive or even antagonistic, which highlights the need to

control drug ratios being exposed to tumor cells (Mayer et al., 2006; Tardi et al., 2007).

In in vitro cell culture systems, ratios of drug combinations exposed to tumor cells can be tightly controlled. This is something that upon systemic administration is extremely difficult to achieve, due to different pharmacokinetic profile of each one of the drugs entering the combination. Under the circumstances, tumor cells are therefore exposed to sub-optimal drug ratios with a concomitant loss in therapeutic activity (Mayer et al., 2006; Tardi et al., 2007). Such problem can be overcome upon encapsulation of the drug combination into a delivery system, like PEGylated liposomes, able to maintain the drug ratio from the site of administration until it reaches the tumor cells.

Small interfering RNA (siRNA) are short double stranded RNA molecules, usually with 21–23 nucleotides in each strand, that trigger the cleavage of target mRNA (Dykxhoorn et al., 2003, 2006; Huang et al., 2008; Kumar and Clarke, 2007). Nevertheless, there are many obstacles to the therapeutic use of siRNA, such as enzymatic degradation (Dykxhoorn et al., 2006; Kawakami and Hashida, 2007; Zhang et al., 2006), low cellular uptake (Akhtar and Benter, 2007; Kawakami and Hashida, 2007; Zhang et al., 2006) and rapid renal excretion (Dykxhoorn et al., 2006; Kawakami and Hashida, 2007), features that are responsible for their poor in vivo pharmacokinetic properties. The use of engineered liposomes to carry siRNA allows to protect the nucleic acids from the degradation mediated by nucleases, to achieve sustained plasma concentrations and to promote targeted delivery of the nucleic acids into tumor cells.

Hydrophilic polymers such as polyethylene glycol (PEG) are introduced in the liposome membrane to reduce their aggregation, opsonization and uptake by macrophages of the mononuclear phagocytic system, allowing the enhancement of circulation times in the blood stream and consequently improving pharmacokinetic features (Immordino et al., 2006; Romberg et al., 2008; Ryan et al., 2008).

The cell surface transferrin receptor (TrfR) has been explored as a target to deliver liposomes into cancer cells (Chiu et al., 2006; Visser et al., 2005), due to its overexpression on tumor cells (Daniels et al., 2006a,b; Li and Qian, 2002), accessibility on the cell surface, and constitutive endocytosis (Daniels et al., 2006a). Transferrin (Trf), an 80 kDa glycoprotein, is the natural ligand for this TrfR (Daniels et al., 2006a; Ponka and Lok, 1999).

Thus, the present work aimed at developing sterically stabilized liposomes targeted to the TrfR and co-encapsulating imatinib and siRNA designed to specifically silence the *BCR-ABL* oncogene. The potential therapeutic advantages achieved with the new formulations co-encapsulating imatinib and siRNA as well as pre-sensitization of tumor cells with imatinib followed by targeted gene silencing, were also evaluated.

For this purpose, different ratios of siRNA/imatinib were tested and their effect on the encapsulation yield of each molecule was evaluated. In parallel, the anti-leukemia activity of the different formulations encapsulating siRNA and imatinib at different ratios and of the combination of Trf-liposomes carrying siRNA and free imatinib under two different treatment schedules of pre-sensitization was also assessed in imatinib-resistant or -sensitive leukemia cells.

Materials and Methods

Chemicals

Cholesterol (Chol) was obtained from Sigma (St. Louis, MO), all the other lipids N-palmitoyl-sphingosine-1-[succinyl(methoxypolyethylene glycol) 2000] (C_{16} $mPEG_{2000}$ ceramide); 1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine (DSPC);1,2-dioleoyl-3-dimethylammonium-propane (DODAP); 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-N-[maleimide (polyethylene glycol)2000] ammonium salt (DSPE-PEG-MAL) were obtained from Avanti Polar Lipids (Alabaster, AL). Human holo-transferrin (Trf) was purchased from Sigma. Anti-*BCR-ABL* siRNA was purchased from Dharmacon RNA Technologies (Lafayette, CO), the antisense sequence is the following 5′AAGGGCUUUUGAACUCUGCdTdT3′, the complementary sense strands exhibit dTdT overhangs. Imatinib mesylate (Glivec®), kindly provided by Novartis Pharmaceuticals (Basel, Switzerland) was dissolved in water and stored at −20°C. All the other chemicals were obtained from Sigma unless stated otherwise.

Preparation of Trf-Coupled PEG_{2000}-DSPE Micelles

Coupling of Trf to PEG_{2000}-DSPE micelles was performed accordingly to Ishida et al. (1999). Briefly, Trf protein was modified with the addition of thiol groups through reaction with 2-iminothiolane hydrochloride (2-IT). For this purpose, Trf and 2-IT freshly dissolved in HEPES buffer (20 mM HEPES, 145 mM NaCl, pH 8) were mixed in a protein/2-IT molar ratio of 1:10 and gently stirred for 1 h, in the dark at room temperature.

A lipid film of DSPE-PEG-MAL was prepared by solvent evaporation under a mild stream of N_2 and further dried under vacuum for 2 h. This dried lipid film was then hydrated with MES buffer (20 mM HEPES, 20 mM MES, pH 6.5), at a concentration above 2.3 μM, the critical micellar concentration of the lipid (Ishida et al., 1999). Micelles were formed by strong vortex followed by 15 s heating in a water bath at 38°C, followed by a second vortex shaking. Then, the freshly thiolated protein was coupled to the freshly prepared DSPE-PEG-MAL micelles by a thioesther linkage (protein to micelles molar ratio of 1:1). The coupling reaction was performed overnight, in the dark at room

temperature with gentle stirring. The remaining free MAL groups in the micelles were quenched by the addition of β mercaptoethanol at a maleimide: β mercaptoethanol molar ratio of 1:5, under stirring for 30 min at room temperature.

Encapsulation of siRNA Into Liposomes

A solution containing 13 μmol of total lipid composed of Chol/DSPC/DODAP/mPEG 2000C_{16}Ceramide (45:22:25:8, mol%) in 200 μL of absolute ethanol, and a solution of 0.041 μmol of siRNA in 300 μL of 20 mM citrate buffer, pH 4, were heated at 60°C. The lipids were then slowly added under strong vortex to the siRNA solution. In some experiments, empty liposomes were used. In this case, lipids were added to 300 μL of citrate buffer under similar conditions as those used for siRNA-encapsulating liposomes preparation. Upon their formation the liposomes were extruded, 21 times, in a LipoFast mini extruder (Lipofast, Avestin, Toronto, Canada) through 100 nm diameter polycarbonate filters (Avestin, Toronto, Canada). Then, a dialysis was performed in HBS, pH 7.4, through regenerated cellulose tubular membrane with MWCO 6000–8000 (Cellu Sep T2, Membrane Filtration Products, Inc., Seguin, TX) during 3 h at room temperature to remove ethanol and raise the external pH. Subsequently, the total lipid concentration was assessed by cholesterol quantification. For this purpose, samples were added to absolute ethanol (1:6, v/v) and Infinity™ Cholesterol Liquid Stable Reagent (Thermo Electron, Melbourne, Australia). Absorbance was measured at 500 nm in a spectrophotometer and the concentration assessed against a cholesterol standard curve. The cholesterol quantification allowed the determination of the total lipid that remained at this stage and consequently the determination of the amount of imatinib and micelles to be added.

Encapsulation of Imatinib in siRNA-Containing Liposomes and Post-Insertion of Trf at the Liposome Surface

Immediately after the dialysis of the liposomes containing siRNA (performed as described previously), imatinib was encapsulated into the liposomes, by addition of imatinib to the siRNA-containing liposomes at different imatinib/total lipid molar ratios (1:3, 1:8, 1:16, 1:32, 1:42; initial imatinib/total lipid) and incubation for 1 h, at 60°C, in a water bath. The liposomes were then allowed to reach the room temperature and 4 mol% of Trf-micelles was added and incubated for 17 h, at 38°C, in a water bath under dark.

Purification of Liposomes

After incubation with micelles, Trf-liposomes were purified by size exclusion chromatography on a Sepharose CL-4B column, using HBS, pH 7.4, as running buffer to remove external siRNA and imatinib as well as chemical reagents used during the liposomal preparation.

SiRNA Quantification

The amount of siRNA entrapped inside liposomes was assessed by the Quant-iT RiboGreen RNA Assay (Molecular Probes, Invitrogen, Karlsruhe, Germany) against a siRNA standard curve. Liposomes were dissolved upon addition of 0.6 mM of octaethylene glycol monododecyl ether ($C_{12}E_8$) and the RiboGreen fluorescence (λ_{ex} 485 nm, λ_{em} 530 nm, cut off 515 nm) was measured using a Spectra Max Gemini EM plate reader fluorimeter (Molecular Devices, Sunnyvale, CA).

Imatinib Quantification

The method for imatinib quantification was developed by adaptation of the Dharmacon RNA Technologies protocol for siRNA precipitation. In microfuge tubes, 0.1 μmol of total lipid was added to the precipitation reagent (400 μL destilled water, 40 μL of 10 M ammonium acetate, pH 7 and 1.5 mL absolute ethanol) up to 800 μL, samples were then submitted to 30 s of strong vortex and transferred to −80°C/2 h or −20°C/overnight. Subsequently, frozen samples were slightly thawed at room temperature and centrifuged at 18,000*g* for 20 min at 4°C. Imatinib concentration was determined in the supernatant by measuring the absorbance at 259 nm against a standard curve of imatinib. This quantification method was optimized to eliminate any interference by the other components of the formulation.

Cell Lines

Two human chronic myeloid leukemia cell lines in blast crisis, positive for *BCR-ABL* oncogene, with the b3a2 translocation (K562 and LAMA-84 cells) purchased from DSMZ (Braunschweig, Germany) were maintained in culture at 37°C, 5% CO_2 under humidified atmosphere in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Gibco, Invitrogen, Carlsbad, CA), penicillin (100 U/mL), and streptomycin (100 μg/mL) (Cambrex, East Rutherford, NJ).

Development of Imatinib-Resistant Cell Line

K562 cells maintained in culture as previously described, were incubated with increasing concentrations of imatinib, starting at 0.05 μM and with 0.05 μM increments every 4 days of culture, until cells acquired the ability to grow at 1 μM. At this time point, drug resistance was assessed and cells were designated as IRK562. The new cell line was maintained continuously in culture in the presence of 1 μM

of imatinib and was washed with drug-free medium before all experimental procedures.

Cell Transfection

K562 and LAMA-84 cells (20,000 cells/well) and IRK562 cells (25,000 cells/well) in RPMI-1640 culture medium supplemented with 10% FBS and antibiotics were seeded in 96-round well plates. Cells were transfected with Trf-associated liposomes co-encapsulating siRNA and imatinib at different molar ratios at 37°C for 4 h. After incubation with liposomes, the medium was replaced with fresh medium and cells further incubated for 44 h. Concerning to the pre-sensitization assays, LAMA-84 cells were seeded as previously described and treated with two different combination schedules of different concentrations of free imatinib with 1 μM of anti-*BCR-ABL* siRNA encapsulated in Trf-coupled liposomes: (i) cells were treated for 48 h with different concentrations of free imatinib, imatinib was removed and 1 μM of anti-*BCR-ABL* siRNA encapsulated in Trf-coupled liposomes was added to cells and the resulting suspension was transferred to 48-well plates; after 4 h of incubation, 800 μL of culture medium was added to each well and cells were kept in culture for 44 h (free imatinib → encaps siRNA), (ii) cells were treated for 4 h with anti-*BCR-ABL* siRNA encapsulated in Trf-coupled liposomes, siRNA was removed and cells were kept in culture for 44 h; different concentrations of free imatinib were further added to cells and the resulting suspension was transferred to 48-well plates and kept incubating for 48 h (encaps siRNA → free imatinib).

Cell Viability

Cell viability was evaluated by the resazurin reduction assay (O'Brien et al., 2000). The assay measures the chemical reduction of the resazurin dye resulting from cellular metabolic activity, and allows the determination of viability over the culture period without harming the cells. Briefly, the culture medium was replaced with 10% (v/v) resazurin dye in RPMI-1640 medium without serum and antibiotics, which was added to each well. After 2.5 h of incubation at 37°C, the absorbance at 540 nm (reduced form) and 630 nm (oxidized form) was measured in a microplate reader Multiskan Ex (Thermo Labsystems, Vantaa, Finland). Cell viability was calculated as percentage of control cells using the equation:

$$\frac{(A_{540} - A_{630})\text{treated cells} \times 100)}{(A_{540} - A_{630})\text{control cells}}$$

Assessment of Imatinib IC_{50} and DRI

The required drug concentration to promote reduction of 50% in cell viability (IC_{50}) and dose reduction index (DRI), which is the magnitude of dose reduction allowed for a drug when given in a drug combination (IC_{50} of the drug when administered alone/IC_{50} of the drug when administered in the combination) were assessed. For IC_{50} determination non-linear curve fit assuming sigmoidal dose-response was performed.

Statistical Analysis

All data are presented as mean ± standard deviation (SD), and are the result of at least three independent experiments. One Way ANOVA analysis of variance combined with Tukey post-test was used for multiple comparisons. Statistical differences are presented at probability levels of $P > 0.05$, $P < 0.05$, $P < 0.01$, and $P < 0.001$.

Results

Evaluation of the Co-Encapsulation Yields of Imatinib and siRNA in Trf-Liposomes

In this work, Trf-coupled sterically stabilized liposomes co-encapsulating imatinib and anti-*BCR-ABL* siRNA in different molar ratios were engineered by modification of the liposome preparation technique developed previously by Mendonca et al. (2010). Thus, after the encapsulation of siRNA in SNALP liposomes, a transmembrane pH gradient between the aqueous content of the liposome (citrate buffer, pH 4) and the external liposome milieu (HBS, pH 7.4) was generated, this being the driving force for the active encapsulation of the imatinib. Trf-PEG-DSPE conjugates were then inserted onto the liposomes by the post-insertion method. Using this methodology, the effect of imatinib on siRNA encapsulation yields (and vice versa) was assessed upon incubation with different imatinib/lipid ratios (1:3; 1:8; 1:16; 1:32; 1:42). As illustrated in Figure 1A, the encapsulation yields of imatinib increased with decreasing of imatinib/total lipid ratios, being 11.88 ± 2.09% for the 1:3 ratio and of 19.8 ± 2.32% for the 1:8 ratio. For ratios above 1:16, the encapsulation yields were very similar, being around 25%. For ratios exhibiting the same yield, the higher loading of imatinib was obtained with increasing imatinib/lipid ratios (i.e., 1:16 > 1:32 > 1:42) (Table I). It is interesting to notice that the presence of siRNA significantly enhances the imatinib encapsulation yields. As can be observed at the ratio of 1:8, the encapsulation yields of imatinib increased from 5.96 ± 2.39%, in liposomes without siRNA, to 19.80 ± 2.32% when imatinib is co-encapsulated with siRNA.

As can be observed in Figure 1B, the siRNA encapsulation yield was significantly affected by the presence of imatinib, namely by the imatinib/total lipid ratio used in the co-encapsulation process. Thus, the formulations prepared with higher amounts of imatinib (higher imatinib/total lipid ratios, e.g., 1:3) resulted in lower siRNA encapsulation

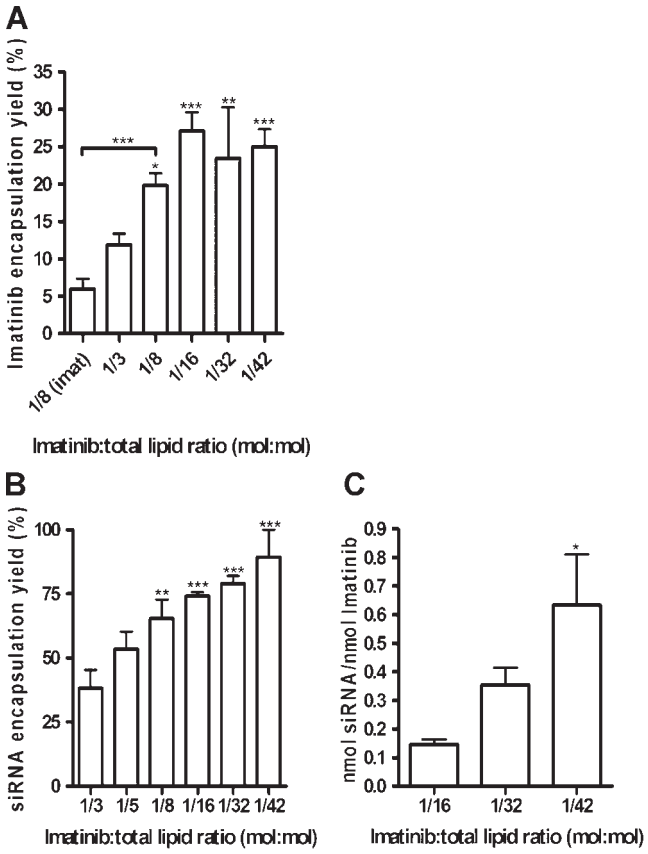

Figure 1. Encapsulation yields of imatinib and siRNA and siRNA/imatinib molar ratio in Trf-liposomes co-encapsulating both molecules. Imatinib was incubated with SNALP liposomes loaded with anti-*BCR-ABL* siRNA under different imatinib/total lipid molar ratios (1:3; 1:5; 1:8; 1:16; 1:32; 1:42), as described in the Materials and Methods Section. In addition, imatinib was incubated with Trf-liposomes without siRNA at 1:8 imatinib/total lipid ratio [referenced in the figure as 1:8 (imat)]. After liposome purification, the final siRNA and imatinib were quantified and the encapsulation yield of imatinib (**A**) and of siRNA (**B**) as well as the siRNA/imatinib molar ratio (**C**) were assessed. No symbol $P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$ when the comparison was established with the 1:3 formulation or between the conditions indicated by the lines (A and B) or with the 1:16 formulation (C).

yields, as compared to the formulations prepared with lower amounts of imatinib (lower imatinib/total lipid ratios, e.g., 1:42). Our previous results (Mendonca et al., 2010), clearly indicate that when siRNA is encapsulated alone, under the same conditions as those in this co-encapsulating process, the encapsulation yield is $92.17 \pm 1.39\%$. Overall, these results demonstrate that when imatinib is co-encapsulated with siRNA, the siRNA encapsulation yield decreases in a manner dependent on the imatinib/total lipid ratio pre-incubated with the liposomes. However, for ratios lower than 1:16, no significant difference in the siRNA loading of the formulations prepared was observed (Table I). Nevertheless, the imatinib loading was smaller for the lower ratios (Table I), allowing to reach higher siRNA/imatinib ratios (Fig. 1C). Thus, the lowest imatinib/lipid ratio tested (1:42) resulted in the highest siRNA/imatinib ratio (0.63). At this ratio it was therefore possible to obtain therapeutic concentrations of both imatinib and siRNA inside the same liposome. In contrast, the 1:3 ratio at which the imatinib loading is too high as compared to that of siRNA, did not allow to reach therapeutic concentrations for both agents. Trf-liposomes co-encapsulating imatinib and siRNA molecules prepared from imatinib/total lipid ratios of 1:16, 1:32 and 1:42 (1:16; 1:32 and 1:42 formulations), resulted in 0.15, 0.35 and 0.63 siRNA/imatinib molar ratio, respectively (formulations are codenamed by the resulting siRNA/imatinib molar ratios).

Table I. Imatinib and siRNA loading parameters of Trf-liposomes co-encapsulating both molecules.

Imatinib/total lipid ratio	Imatinib (nmol/μmol TL)	siRNA (nmol/μmol TL)
1:16	20.91 ± 7.0	3.19 ± 1.49
1:32	9.70 ± 4.42	3.38 ± 1.54
1:42	5.99 ± 0.82	3.90 ± 2.02

Trf-liposomes co-encapsulating imatinib and siRNA were formulated from three different imatinib/total lipid initial ratios (1:16; 1:32; 1:42) and the loading of each one of the encapsulated molecules (imatinib and siRNA) was assessed as described in the Materials and Methods Section. TL, total lipid.

Cytotoxicity of Trf-Liposomes Co-Encapsulating siRNA and Imatinib at Different Molar Ratios

The formulations that allowed to obtain therapeutic concentrations for both imatinib and siRNA within the same liposome were tested against cell lines sensitive to imatinib (K562 and LAMA-84) as well as against the imatinib-resistant IRK562 cell line. As can be observed in Table II, for all tested cell lines the formulation with higher amount of anti-*BCR-ABL* siRNA (1:42) led to higher imatinib IC_{50} reduction and, consequently, to higher dose reduction index (DRI). IRK562 cells were more sensitive to the increment of the siRNA dose in the combination of siRNA and imatinib than the imatinib-sensitive cell line LAMA-84, since for the 1:16 and 1:42 formulations the DRI was of 1.16 and 3.84, respectively, whereas for LAMA-84 the difference between the DRI obtained for the different formulations was not so evident. In fact, lower siRNA/imatinib ratios were required to reach the same DRI in LAMA-84 cells as compared to K562 and IRK562 cells, indicating that cell lines with higher *BCR-ABL* oncogene levels are more dependent on the gene silencing agent concentration. *BCR-ABL* mRNA levels followed the order IRK562 > K562 > LAMA-84 as assessed by qRT-PCR (data not shown).

LAMA-84 cells were also treated with different concentrations of free imatinib combined with 1 μM of siRNA encapsulated in Trf-liposomes (Table II). Such treatments required higher siRNA/imatinib ratios than that required by the strategy in which both molecules are co-encapsulated in the same liposome. In fact, for the combination of imatinib and encapsulated siRNA, the siRNA/imatinib ratios used were 2.5–200, which led to a 2.85 DRI, whereas

Table II. Effect of Trf-liposomes loading different siRNA/imatinib molar ratios on imatinib IC_{50} and DRI, in imatinib-resistant and non-resistant leukemia cells.

	siRNA/imatinib (imatinib/lipid)					
Cell line	0.15 (1:16)	0.35 (1:32)	0.63 (1:42)	Free imatinib + siRNA encaps	Free imatinib	Imatinib encaps
IRK562						
IC_{50} (μM)	3.03 ± 0.95	1.49 ± 0.19	0.91 ± 0.09	n.a.	3.49 ± 0.32	n.a.
DRI	1.16	2.34	3.84	—	—	—
K562						
IC_{50} (nM)	85.90 ± 6.78	60.40 ± 4.60	n.a.	n.a.	138.0 ± 34.0	122.0 ± 14.0
DRI	1.61	2.28	—	—	—	—
LAMA-84						
IC_{50} (nM)	50.40 ± 4.8	36.80 ± 2.50	35.60 ± 4.2	54.10 ± 16.20	84.0 ± 8.0	70.0 ± 4.30
DRI	2.42	3.32	3.43	2.85	—	—

IRK562, LAMA-84 and K562 cells were treated with Trf-liposomes co-encapsulating anti-*BCR-ABL* siRNA and imatinib at the molar ratios of 0.15, 0.35, and 0.63, which correspond to the formulations prepared from imatinib/total lipid molar ratios of 1:16, 1:32, and 1:42, respectively. Cells were also treated with non-encapsulated imatinib (free imatinib), imatinib encapsulated in Trf-liposomes (imatinib encaps) or with the combination of different concentrations of free imatinib with 1 μM of anti-*BCR-ABL* siRNA encapsulated in Trf-liposomes (free imatinib + siRNA encaps). Treatments took place for 48 h and then cell viability was assessed. For IRK562 cells treated with siRNA co-encapsulated with imatinib in Trf-liposomes, siRNA molecules were used at 0.32–1.58 μM for 0.63 formulation; 0.18–1.77 μM for 0.35 formulation and 0.075–1.50 μM for 0.15 formulation. For K562 and LAMA-84 cells the siRNA was used in the range of 3.16 nM to 0.25 μM for 0.63 formulation; 1.78 nM to 0.14 μM for 0.35 formulation and 0.77 nM to 0.061 μM for 0.15 formulation. All the encapsulated anti-*BCR-ABL* siRNA doses used in the combination protocols were also tested in the absence of imatinib, and did not show any cytotoxicity. DRI, dose reduction index; n.a., not assessed.

with the 1:42 formulation, in which siRNA/imatinib ratio is 0.63, a higher DRI (3.43) was obtained. Results indicated that free imatinib and imatinib encapsulated in Trf-liposomes promote similar cytotoxicity, suggesting that these Trf-coupled liposomes loading imatinib allow efficient intracellular drug delivery (Table II).

Effect of the Administration Schedule of Free Imatinib and Encapsulated siRNA on Cell Viability

It is known that for some drug combinations, the pre-sensitization of cells with a first drug enhances the response to the second drug. Therefore, in this work the effect of two different treatment schedules based on the combination of anti-*BCR-ABL* siRNA encapsulated in Trf-liposomes and free imatinib was evaluated. As can be observed in Table III, the two schedules led to significant DRI. The highest DRI (8.71) was observed for the schedule in which the cells were first treated for 48 h with siRNA and then treated for more 48 h with imatinib. The schedule involving a pre-sensitization with imatinib for 48 h followed by 48 h of treatment with siRNA led to a DRI of 2.16. Treatment of the cells with siRNA encapsulated in Trf-liposomes at the same concentrations as that used in the combination treatments had no significant effect on cell viability (92.11 ± 1.57%), (data not shown).

Table III. Effect of the treatment schedules of free imatinib and Trf-liposomes loaded with anti-*BCR-ABL* siRNA on the imatinib IC_{50} and DRI in LAMA-84 cells.

	IC_{50}	DRI
(i) Free imatinib → encaps siRNA (nM)	19.80 ± 5.17	2.16
(ii) Encaps siRNA → free imatinib (nM)	4.90 ± 1.40	8.71
(iii) Imat (96 h) (nM)	42.70 ± 9.50	—

(i) LAMA-84 cells were treated with different concentrations of free imatinib for 48 h, imatinib was removed and 1 μM of anti-*BCR-ABL* siRNA encapsulated in Trf-coupled liposomes was incubated for 48 h (free imatinib → encaps siRNA). (ii) The previous treatment given in reverse order (encaps siRNA → free imatinib). (iii) The cytotoxicity of non-encapsulated imatinib after 96 h of incubation was also assessed [imat (96 h)].

Discussion

Despite all the progress achieved over the last decades on understanding carcinogenesis and on the development of more specific anti-tumor therapies, there is still much to be done in cancer treatment. Treatment schedule with combination of multiple drugs is a common practice in the treatment of cancer and enables responses impossible to obtain with single-agent therapies (Blagosklonny, 2005; Li et al., 2006; Wang et al., 2005; Wu et al., 2007; Zimmermann et al., 2007; Zoli et al., 2001). However, the pharmacological interaction between drugs can be synergistic, additive or antagonistic, depending on the drug molar ratios used in the combination, therefore dramatically affecting the antitumor activity (Abraham et al., 2004; Tardi et al., 2007).

The control of combined drug ratios exposed to tumor cells upon systemic administration is therefore of great relevance. Nevertheless, it should be emphasized that it is very difficult to control the pharmacokinetics of each of the drugs utilized in a drug combination protocol, namely in what concerns their ability to reach target tissues or cells at a certain molar ratio. A successful strategy to overcome this limitation is the development of liposomes co-encapsulating two or more drugs at the desired molar ratio (Abraham et al., 2004; Mayer et al., 2006; Tardi et al., 2007). In this regard, the encapsulation protocol and lipid composition

represent key factors to ensure efficient and stable encapsulation of the drugs, namely when intravenous administration is envisaged.

One of the aims of this work was the development of liposomes allowing triple-targeting, including cellular targeting and molecular targeting at two different levels within the tumor cell. Thus, transferrin receptor-targeted sterically stabilized liposomes co-encapsulating anti-*BCR-ABL* siRNA and imatinib were developed. For this purpose, the formulation of TrfR-targeted liposomes encapsulating siRNA developed by Mendonca et al. (2010) was adapted for co-encapsulating siRNA and imatinib. Our previous work clearly indicated that TrfR-targeted liposomes deliver intracellularly the siRNA and asODN by a Trf receptor-dependent process as opposed to what happened with the non-targeted counterpart that did not reveal any capacity whatsoever to deliver the nucleic acids. It was also demonstrated that the TrfR-targeted liposomes encapsulating siRNA promoted sequence-specific downregulation of the *BCR-ABL* mRNA. Thus, the present work is centered on the co-encapsulation of siRNA and imatinib and further advantages in terms of leukemia cells cytotoxicity achieved with the developed formulations.

Therefore, this work aimed at the development of liposomes co-encapsulating anti-*BCR-ABL* siRNA and imatinib in sterically stabilized liposomes targeted for TrfR. For this purpose, the siRNA encapsulation procedure based on the electrostatic interaction with ethanol-destabilized cationic liposomes was adopted (Semple et al., 2001). This approach resulted in high siRNA entrapment, which is attributed to the presence of the ionisable cationic lipid DODAP (Mendonca et al., 2010).

For imatinib encapsulation, an active entrapment approach was used. Among the two main well-known procedures to promote active drug loading into liposomes (Abraham et al., 2004; Li et al., 2008), the pH gradient method was selected. This approach relies on the enhanced permeation ability of the neutral form of the drug which allows it to cross the liposomal membrane and be entrapped in the liposomal lumen due to its protonation at acidic pH (imatinib has 4 protonable amine functions (Szakacs et al., 2005)), thus leading to efficient drug loading (Cullis et al., 1997; Saxon et al., 1999).

Encapsulation of imatinib into liposomes has been performed by others (Beni et al., 2006; Harata et al., 2004). However, to the best of our knowledge, this is the first report of co-encapsulation of small molecular weight drugs and nucleic acids, both components being encapsulated through active encapsulation procedures. Following loading of the liposomes with the therapeutic agents, their surface was engineered by post-insertion of Trf-PEG-DSPE conjugates, aiming at conferring targeting properties.

The yields of imatinib and siRNA encapsulation, as well as the influence that each of these molecules has on the encapsulation of the other was assessed. Low encapsulation yields of imatinib were observed, which can be attributed to the fact that at pH 7.4 (liposomal external pH) a third of the drug is at the monocationic form (Szakacs et al., 2005), and, therefore, not available to permeate the liposome membrane. This imatinib encapsulation yield dependency on the pH was also observed by others (Beni et al., 2006), clearly demonstrating that the encapsulation yields of the neutral form of imatinib are higher than those of the monocationic form. In addition, it is also reasonable to consider that the weak buffering capacity of the 20 mM citrate buffer used may also contribute to the low encapsulation yields. The buffer capacity is known to be an important factor in the encapsulation efficiency (Cullis et al., 1997; Saxon et al., 1999) and usually, a 300 mM concentration of this buffer is used to generate a pH gradient (Saxon et al., 1999). In this study, 20 mM citrate buffer was used because, as demonstrated from the results presented previously (Mendonca et al., 2010), with 300 mM citrate buffer the siRNA encapsulation is extremely low. Therefore, for cost-effectiveness reasons, experimental conditions were adjusted to favor nucleic acid encapsulation yields.

Surprisingly, the presence of siRNA enhances the imatinib encapsulation yields. This can be explained by interactions between the negatively charged siRNA and the positively charged imatinib (at the intra-liposomal milieu), which may increase the amount of imatinib entrapped inside the liposomes. In what concerns to the role of imatinib in the siRNA entrapment yield, a remarkable decrease in the siRNA yield of encapsulation was observed for the highest ratios of imatinib/total lipid, whereas no impact was observed for the lower imatinib/lipid ratios, as compared to the encapsulation of siRNA in the absence of imatinib. A possible explanation is that the imatinib that remains uncharged permeate the liposomal membrane, and then undergo protonation, thus being retained inside the liposomes. As imatinib extensively sequesters protons in the liposomal lumen, DODAP molecules tend to become neutral, thus leading to detachment of siRNA molecules from the liposomal membrane and leakage events. Alternatively, the positively charged imatinib may interact electrostatically with siRNA molecules forming siRNA/imatinib complexes. The formation of these siRNA/imatinib complexes, together with the destabilization of the liposomal membrane promoted by the post-insertion of micelles may contribute for a significantly destabilization of the liposomal membrane, allowing the leakage of the siRNA that is not bound to the liposomal membrane. Thus, we speculate that by cumulative mechanisms, imatinib has the ability to reduce the amount and strength of siRNA bound to the inner liposomal membrane and that membrane destabilization will facilitate leakage of siRNA. Thus, as observed by others, we have shown that the inclusion of a second drug may induce leakage of the first encapsulated drug (Saxon et al., 1999; Waterhouse et al., 2001), in a drug amount-dependent manner.

Concerning the effect of the developed formulations on cell viability, only the 1:16, 1:32 and 1:42 formulations were tested in vitro, since only these were able to exhibit therapeutically relevant siRNA/imatinib ratios (0.15; 0.35,

and 0.63, respectively). In opposition to the 1:3 and 1:8 formulations for which the achievement of therapeutic concentrations for siRNA will lead to extremely cytotoxic imatinib concentration, which would block the possible therapeutic contribution from the siRNA molecules. Therefore, one can assume that a stronger contribution of the anti-*BCR-ABL* siRNA with respect to imatinib to the induced cell toxicity is observed for the 1:42 formulation.

For all tested cell lines, the 1:42 formulation (highest relative siRNA contribution in the drug combination) led to the highest imatinib IC_{50} reduction, demonstrating the importance of achieving a level for both molecules within the range of therapeutic concentrations. Results also revealed that the imatinib-resistant cell line IRK562 required higher siRNA/imatinib ratios, as compared to non-resistant cell line LAMA-84. In order to better evaluate the response of the different cell lines to the different formulations tested, the levels of Trf receptor at the cell surface (which is the cellular entrance gate for the formulations) were assessed by CD71 quantification by flow cytometry (data not shown). Our results indicate different levels of expression as a function of the cell line: LAMA-84 > K562 > IRK562. Additionally, the *BCR-ABL* mRNA levels also differ considerably between the three cell lines, IRK562 > K562 > LAMA-84, as assessed by qRT-PCR (data not shown). Our results indicate that a correlation between the cellular response and the expression of Trf receptor and *BCR-ABL* mRNA levels could be established. In fact, the cell line with higher Trf receptor expression and lower *BCR-ABL* mRNA levels (LAMA-84) demonstrated higher response to the tested formulations. Therefore, even considering that other cellular features may play relevant roles in the response to targeted therapy, our results strongly suggest that both the levels of Trf receptors and *BCR-ABL* mRNA can be used as biomarkers to predict the efficacy of the developed therapies. It was also observed, that upon co-encapsulation of siRNA and imatinib in the same liposomal particle, lower siRNA/imatinib ratios can be employed to induce a certain degree of cytotoxicity as compared to the co-treatment of the cells with encapsulated siRNA and free imatinib. This reinforces the need of controlling the drug ratio at the intracellular level, demonstrating the importance of co-encapsulating the drugs for the successful application of this therapeutic approach. The treatment of the cells with the drugs delivered by separate liposomes would render very difficult to predict and control the release rate of each drug inside the cells and thus to obtain the desired molar ratios intracellularly. Moreover, delivering drugs in distinct liposomes would require the use of higher lipid concentrations which may induce non-specific cytotoxic effects.

It has been demonstrated that pre-sensitization of leukemia cells with gene silencing agents can enhance the therapeutic effect of imatinib (Aichberger et al., 2005; Carter et al., 2006) and that the pre-sensitization with imatinib enhances radiotherapy and chemotherapy effects (Yerushalmi et al., 2007). Therefore, in order to assess if pre-sensitization of CML cells with imatinib or siRNA followed by treatment with siRNA or imatinib, respectively, could result in an enhancement of cell toxicity, the effect of two different protocols of pre-sensitization were tested. As free siRNA molecules exhibit inefficient cellular uptake, delivery of these molecules was accomplished upon encapsulation in Trf-coupled liposomes. Imatinib was administered in the free form, since as our results demonstrated liposome encapsulation does not result in an enhancement of its in vitro toxicity. Thus, the effect of combining free imatinib and Trf-coupled sterically stabilized liposomes encapsulating siRNA on the viability of LAMA-84 cells was assessed. Our results indicate that pre-sensitization of the cancer cells with siRNA during 48 h followed by 48 h of treatment with imatinib is a more promising therapeutic schedule, associated to a higher DRI, as compared to pre-sensitization with imatinib followed by cell incubation with siRNA. Thus, as demonstrated in our previous work (unpublished data) and by others (Azzariti et al., 2004; McHugh et al., 2007; Zupi et al., 2005), the treatment efficiency of combinatory strategies is schedule-dependent. The higher imatinib IC_{50} fold reduction observed when cells were pre-sensitized with anti-*BCR-ABL* siRNA can be explained by the fact that the over expression of the Bcr-Abl oncoprotein is known to be an important mechanism leading to imatinib-resistance. Taking into consideration the specificity associated to the mechanism of action of imatinib, it is expected that down regulation of the Bcr-Abl oncoprotein, by decreasing the respective *BCR-ABL* mRNA levels, would result in an increased sensitivity of the leukemia cells to imatinib, given that, there is less amount of the oncoprotein to be inhibited. In addition, it is reasonable to consider that down regulation of Bcr-Abl oncoprotein may impair other oncogenic related mechanisms also involved in altered differentiation, apoptotic and proliferation processes, thus rendering the cells more prone to the cytotoxic effect of drugs like imatinib.

As cell viability experiments with co-encapsulated therapeutic agents were only conducted up to 48 h and the pre-sensitization experiments were conducted for 96 h, it remains to be clarified whether co-encapsulation or sequential addition protocols would lead to better results. However, even though promising results were obtained with the sequential treatment, major difficulties are expected when translating this approach to in vivo, namely due to limitations to assure that the therapeutic concentration of each drug is achieved at the level of target cells. In contrast, the co-encapsulation approach is based on the maintenance of the drug/siRNA ratio at the intracellular level and, therefore, encompasses a much higher potential for application in clinics.

Conclusion

The present work illustrates a novel technological approach that renders possible the implementation of a triple targeting

strategy, based on the use of a single therapeutic agent. In fact, the developed sterically stabilized liposomes co-encapsulating imatinib and siRNA against *BCR-ABL* are targeted to transferrin receptors at the cancer cell surface addressing two different molecular targets, *BCR-ABL* mRNA and Bcr-Abl protein. Moreover, the developed liposomes enable the encapsulation of both siRNA and imatinib at molar ratios that allow reaching therapeutic doses, which clearly resulted in increased anti-tumoral activity. Altogether, our results indicate that the developed formulations co-encapsulating different siRNA/imatinib ratios and the pre-sensitization strategies are very promising therapeutic approaches that can fulfill some therapeutic gaps resulting from drug resistance phenomena.

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