

Effectiveness of Free and Liposome-Entrapped Antitumoral Drugs against Hepatocellular Carcinoma: A Comparative *In vitro* Study

André da Silva Gomes^{1,4,†}, Filipe Magno Pascoal Reis^{2,4,†}, Isabela Penna Ceravolo³,
Marcus Vinícius Dias-Souza^{4,*} 

¹ Oncohematology department, Oncobio Hospital, Belo Horizonte, Minas Gerais, Brazil

² Medical BSc Program, University Vale do Rio Doce, Governador Valadares, Minas Gerais, Brazil

³ René Rachou Institute, Belo Horizonte, Minas Gerais, Brazil

⁴ Reference Center for Drug Information (CRIMED), Pitágoras College, Ipatinga, Minas Gerais, Brazil

† Authors contributed equally to this work.

* Correspondence: souzamvd@gmail.com (M.V.D.S.);

Scopus Author ID: 56543795600

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Abstract: Hepatocellular carcinoma (HCC) is a highly prevalent form of liver cancer. Sorafenib is the first-line treatment for patients who are not eligible for surgical procedures. Still, it causes important adverse effects, does not provide symptoms relief, and is marketed at expensive costs. Here we investigated the effectiveness of imatinib, sunitinib, and bortezomib against an *in vitro* model of HCC. These drugs are less expensive than Sorafenib and are approved for other malignant neoplasia. The drugs were tested as free and liposome-entrapped forms against HepG2 cells. Liposomes were prepared without using organic solvents and were tested for physicochemical stability. Entrapment efficiency was determined by spectrophotometric methods. Liposomes' average size was 238 ± 33 nm, with an average entrapment efficiency of 34.3%. All drugs significantly decreased the viability of HepG2 cells in their free form and liposome-entrapped forms compared to the control. In spite of the low entrapment efficiency, no organic solvent was used in liposomes preparation, and cell viability values after 18h treatments with liposome-entrapped drugs were only surpassed by the highest concentration of free drugs. Our data open doors for more studies to assess the safety of the liposomes *in vivo*.

Keywords: anticancer drugs; liposomes; HepG2 cells; hepatocellular carcinoma.

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1. Introduction

Liver cancer is one of the leading causes of mortality among cancers worldwide. Hepatocellular carcinoma (HCC) accounts for 90% of all primary liver cell cancers and is the ten most commonly diagnosed malignant diseases [1]. Risk factors for HCC include chronic infection with hepatitis B and C viruses, alcoholism, smoking, nonalcoholic steatohepatitis, and cirrhosis [2]. Patients with HCC may present painful abdominal distension, a palpable mass in the upper abdomen, jaundice, ascites, asthenia, weight loss, cachexia, malaise, and digestive bleeding, often followed by hepatosplenomegaly and/or liver failure [1,3]. The disease is more prevalent in men aged 45 years or older, and the diagnosis requires histological evidence, computed tomography, and/or magnetic resonance imaging [3].

The pathophysiology of HCC involves a multistage process that triggers cellular stress in hepatocytes through different mechanisms, including endoplasmic reticulum injury, DNA

damage, necrosis, and production of reactive oxygen species (ROS), which contribute to genomic instability and activation of oncogenes and/or inactivation of tumor-suppressing genes [4,5]. The death of liver cells creates a microenvironment that supports the proliferation of tumor cells, which present varied genetic and epigenetic alterations [5]. NADPH oxidase (NOX) represents the main non-mitochondrial source of ROS during hepatocarcinogenesis, especially NOX 1, the main biomarker of the negative prognosis of the disease [6].

Treatment options for HCC include surgical interventions, chemical ablation by percutaneous injection of ethanol or acetic acid, radiofrequency ablation, chemoembolization, and radioembolization [7]. Systemic therapy with Sorafenib is the first-line treatment for HCC patients with advanced and irresectable tumors, a multiple tyrosine kinase inhibitors with antiproliferative and antiangiogenic properties [8-10]. Nevertheless, it causes important adverse effects, offers no symptoms relief, and its high cost makes the therapy inaccessible to most patients with HCC, who often are not eligible for resection surgery or transplantation [11].

An important technological advancement in pharmacotherapy, especially for cancer treatment, is the use of liposomes. These are spherical nanovesicles made of phospholipids of single or multiple layers that can be used to encapsulate hydrophilic and/or hydrophobic molecules [12,13]. This advanced controlled release system may improve the pharmacokinetic profile of drugs and prevent undesired effects, as the pharmacological effects may be reached with a significantly lower concentration of entrapped drugs [12,14]. Liposomes are biocompatible, lack toxicity and immunogenicity, and may be modulated to release drugs under specific biological conditions [15,16]. Nevertheless, specific liposomal formulations for HCC treatment are not available yet.

Here we assessed the antiproliferative effect of different antitumor drugs against HCC in an *in vitro* model. Imatinib (indicated mostly for leukemia), sunitinib (renal cell carcinoma), and bortezomib (multiple myeloma) were tested *in vitro* at varied concentrations in free form and as liposome-entrapped anticancer drugs (LEAD) against HepG2 cells. Free drugs and LEAD were effective in decreasing cell viability, and the results observed after 18h treatments with LEAD were only surpassed by the highest concentration of free drugs. The urgency of new treatments for HCC, and the scarcity of studies with these drugs for HCC make our data even more relevant.

2. Materials and Methods

2.1. Drugs.

Imatinib (IMT), sunitinib (SUT), and bortezomib (BTZ) were purchased from Sigma (St Louis, M.O., U.S.A.). Stock solutions of 5 mg/mL were prepared in a sterile deionized water/DMSO warm solution (10:1 v/v) and kept at 4°C in sterile recipients up to the moment of use.

2.2. Liposomes preparation.

Liposomal formulations were prepared as previously described [17], using Phosal 75 SA (Lipoid GmbH, Germany), with some modifications. First, we prepared empty liposomes by diluting Phosal in sterile deionized water at 3:7 v/v ratio under maximum speed stirring (1 h) at home temperature. This system was subjected to two ultrasound cycles (25 KHz, 1h each

cycle, 5 min interval) and extrusion in 450 nm PVDF membranes (Durapore, Merk Millipore, U.S.A., five times). Following, they were mixed to a 100 mM sucrose cryopreservation solution (2:1 v/v) and freeze-dried. The dried liposomes were hydrated with sterile aqueous solutions of each drug at 500 µg/mL for 1 h at 4°C to encapsulate the drugs. Each liposomal system was then vortexed at maximum speed to reach homogeneity and kept at 4°C overnight.

The systems were then centrifuged (20,000g, 4°C, 2h), and the supernatants were reserved for entrapment efficiency experiments. Liposomes pellets were resuspended in sterile saline and subjected to extrusion in 450 and then 220 nm PVDF membranes (10 times each). The resulting suspensions were diluted in 0.9% saline (1:10 v/v) and assessed by dynamic light scattering (Zetasizer Nano ZS, Malvern) to determine the size of the liposomes and the polydispersity index (4.5 mW laser diode, 670 nm, 173° detection angle). Readings were performed in triplicate for each formulation.

2.3. Entrapment efficiency (EE) estimation.

We used an indirect approach to avoid the need for liposome lysis to estimate the EE. The supernatants obtained in the previous subsection were analyzed by spectrophotometry to determine the concentration of free drugs based on calibration curves prepared on the day of the experiments. Wavelengths were used as follows: IMT = 235 nm [18], SUT = 431 nm [19], BTZ = 270 nm [20]. EE percentiles were then calculated based on the differences in the concentrations used to prepare the liposomes and their concentration in each supernatant, as described [17].

2.4. Stability of liposomes.

We assessed the stability of the liposomal formulations by keeping them frozen in the cryopreservation solution at different storage temperatures (0, -20, and -80 °C) for a total of 60 days. At the 30th, 45th, and 60th days of storage, each liposomal formulation was centrifuged, resuspended in sterile saline, and subjected to new antitumoral activity tests. Results were collected and subjected to statistical analysis. If significant differences were noticed, zeta potential analysis would be conducted.

2.5. Cell culture.

HepG2 cells (American Type Cell Culture, USA) were cultured for 18 h in polystyrene flat-bottomed 96-wells plates using the conditions previously described in detail [21], to reach 5×10^3 cells/well. These conditions were adopted to test both free drugs and LEAD.

2.6. In vitro activity of free drugs and LEAD against HepG2 cells.

IMT, SUT, and BTZ stock solutions were used to prepare them in concentrations ranging from 1000 to 0.488 µg/mL by diluting them in sterile warm RPMI media (Merk, USA). A total of 20 µL of the drugs in each concentration was added to the wells with a volume of 180 µL of cell culture. The plates were incubated for 18 h (5% CO₂ atmosphere, 37°C). We used fluorimetric readings with resazurin staining (0.1 g/mL, λ_{ex} 570 nm, λ_{em} 590 nm) as described [17] to assess the viability of treated cells. Arbitrary fluorescence units (AFU) were collected, and the averages were calculated.

Liposomal suspensions were prepared at the concentration of 50 µg/mL by 10-fold dilutions in RPMI media. A total of 20 µL of each LEAD suspension was added to the wells with a volume of 180 µL of cell culture. Plates were incubated for free drugs. Resazurine staining and fluorimetric readings were also performed at 1, 2, 3, 4, 5, 6, and 18 h of treatment. AFU was then collected, and the averages were calculated. All assays were conducted as two independent experiments with triplicates for each concentration of free drugs or LEAD type. Untreated cells were used as a negative control for free drugs. For LEAD, untreated cells and cells treated with empty liposomes were used as negative controls.

2.7. Statistics.

Normality and homoscedasticity of data were checked by Shapiro-Wilk and Bartlett's tests, respectively. Differences in cell viability readings averages following the treatments were analyzed by ANOVA with posthoc Tukey test and considered significant if $p < 0.05$ (or highly significant if $p < 0.01$). These data were analyzed using Bioestat 5.0 for Windows. The effect of time on the results obtained with liposomes was assessed through the Scott-Knott test, using SISVAR for Windows. We calculated the half-maximal inhibitory concentration (IC_{50}) and coefficient of determination (R^2) for the free drugs using GraphPad Prism 9.2.0 for Windows.

3. Results and Discussion

3.1. Characterization of the developed liposomes.

Table 1 summarizes the main characteristics of the liposome formulations. Their average size was 238 ± 33 nm, with a polydispersity index of 0.082 ± 0.010 and an average EE of 34.3%. The average liposome yield was 71.6 ± 9.6 mg.

Table 1. Properties of the liposomes prepared with anticancer drugs.

LEAD	Size (nm)	PDI	EE	ECDL (µg/mg)	Liposome Yield (mg)
IMT	216	0.094	38%	2.71	63 mg
SUT	276	0.073	31%	2.46	70 mg
BTZ	223	0.081	34%	2.07	82 mg

LEAD: liposome-entrapped anticancer drug(s). IMT: Imatinib. SUT: Sunitinib. BTZ: Bortezomib. PDI: polydispersity index. EE: Entrapment efficiency. ECDL: estimated concentration of drugs in liposomes (µg of drug/mg of liposomes). Liposome yield is the total liposomes (in mg) at the final preparation step.

3.2. Antitumoral activity of free drugs.

In all tested concentrations, drug treatments in free form reduced the cellular viability significantly compared to the untreated control (control AFU readings average = 2531, $p < 0.05$, data not shown to keep the lowest values visible on figures). As expected, the highest concentration (1000 µg/mL) was the most effective against HepG2 cells (Figure 1) when compared to the other concentrations ($p < 0.05$).

Although there was no significant difference in the antitumoral activity of the tested drugs ($p = 0.0673$), some tendency of better results was observed for BTZ (Figures 1 A and B – control AFU average was not included to keep the lowest values visible). Compared to the control, BTZ treatment decreased cell viability by 90%, IMT in 89%, and SUT in 87%. The IC_{50} value for BTZ was the lowest found (4.694 µg/mL, $R^2 = 0.8665$), followed by IMT (10.38

$\mu\text{g/mL}$, $R^2 = 0.8926$) and SUT ($12.13 \mu\text{g/mL}$, $R^2 = 0.954$). These results confirm a tendency of better results with BTZ compared to the other drugs.

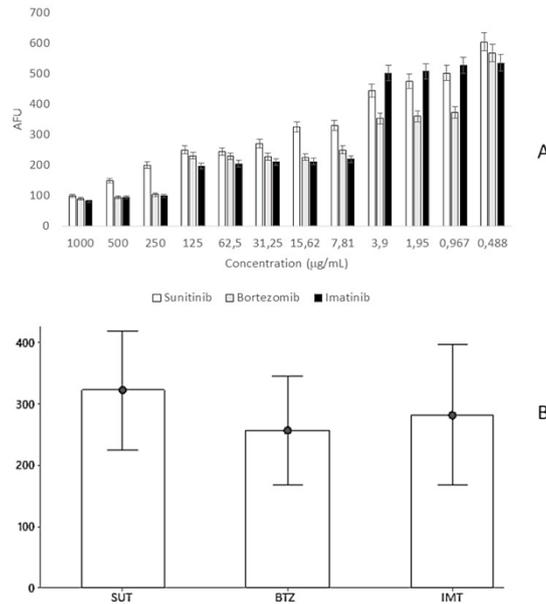


Figure 1. Antitumoral activity of free anticancer drugs. (A) AFU (arbitrary fluorescence units) for each tested concentration following 18h exposure. (B) Average AFU reduction following each drug treatment. SUT: Sunitinib, BTZ: Bortezomib, IMT: Imatinib.

3.3. Antitumoral activity of LEAD.

Freshly-prepared liposomes were tested against the HepG2 cells and were effective and statistically different from the control since the first hour of exposure ($p < 0.05$). The antitumor effect was more pronounced after the 5th hour of treatment for all entrapped drugs. As for the free drugs, no significant effect was detected among the LEAD treatments, but a tendency for better results was observed for BTZ. The 18h AFU values were only surpassed by the highest concentration of free drugs. Considering the lower concentration of the entrapped drugs compared to their free counterparts, these results become even more relevant.

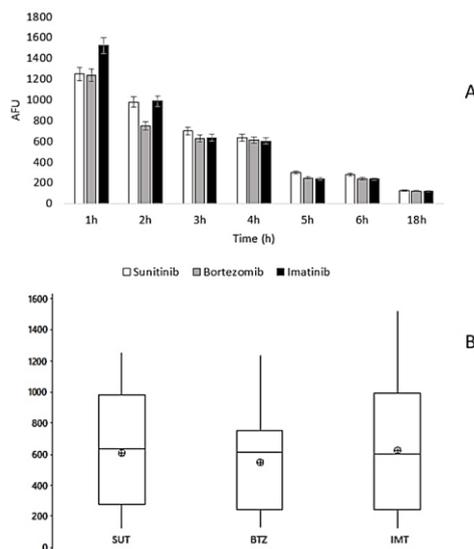


Figure 2. Antitumoral activity of liposome-entrapped anticancer drugs (LEAD). SUT: Sunitinib, BTZ: Bortezomib, IMT: Imatinib. (A) AFU (arbitrary fluorescence units) for each tested LEAD, from 1-18h. (B) Box plot of the AFU reduction of each treatment after 18 h exposure. Points represent the average, and lines represent the median.

When the antitumor activity of the stored liposomes was evaluated and compared to freshly prepared formulations, we noted no statistically significant differences concerning the antitumoral potential in none of the LEAD ($p>0.05$, data not shown) after 30 and 45 days of storage. However, after 60 days, liposomes stored at 0 °C were not as effective as freshly prepared liposomes (Table 2). Zeta potential analysis indicated relevant alterations in the formulations' size and dispersion indexes.

Table 2. Properties of the liposomes after 60 days of storage at 0°C.

LEAD	Size (nm)	PDI	D-AFU-R
IMT	385	0.202	37%
SUT	417	0.105	48%
BTZ	356	0.112	55%

LEAD: liposome-entrapped anticancer drug(s). IMT: Imatinib. SUT: Sunitinib. BTZ: Bortezomib. PDI: polydispersity index. D-AFU-R: decreased arbitrary fluorescence units rate compared to freshly prepared liposomes. All parameters were significantly different from freshly prepared liposomes ($p<0.05$).

None of the tested drugs are currently indicated for the treatment of HCC due to the lack of evidence of their effectiveness. Here we provided evidence of IMT, SUT, and BTZ effectiveness in an *in vitro* model of the disease, with a tendency of better results for free and liposome-entrapped BTZ. There are few studies exploring these drugs for HCC treatment, and to the best of our knowledge, this is the first time that IMT and BTZ liposomes have been tested against HepG2 cells. The current cost of the tested drugs is considerably lower than Sorafenib (gold-standard), and if these results are confirmed in further *in vivo* assays, the use of these drugs for HCC treatment might be possible soon.

The use of liposome-entrapped drugs in cancer treatments provides important therapeutic advantages such as lower frequency of administration and less intense/frequent side effects [12]. The ability of liposomes to release drugs in a more controlled and localized way to cells is even more relevant concerning intracellular targets: cell uptake of drugs might be increased due to the similar biochemical composition of vesicles and cell membranes [12-14]. The liposomal formulations developed in this study presented around 238 nm and low polydispersity indexes and remained effective after being stored in negative temperatures (except 0 °C). The total amount of drugs entrapped in the liposomes was approximately 3 µg/mL (Table 1), and their effectiveness was similar to the free form counterparts at 400-fold higher concentrations after 18 h exposure (Figs 1 and 2). In spite of the low entrapment efficiency, it is important to mention that the method does not require organic solvents, avoiding purification steps and making liposome preparation and the final formulation safer than conventional methods.

Liposomes stored at 0 °C were significantly less effective than the freshly prepared liposomes after 60 days of storage and had relevant size alterations. The increased size of the liposomes can be (at least partially) explained by coalescence, aggregation, or even swelling of the nanovesicles [14,22]. This alteration can be caused by factors that include (but are not limited to) the composition of the liposomes, the type of solution in which they are on, the temperature of storage, and the concentration of vesicles per volume [23,24]. Such alterations were not detected for liposomes kept at -20 and -80 °C, suggesting ideal temperatures for their storage.

IMT inhibits receptor tyrosine kinases and cell proliferation and induces apoptosis of cancer cells [25-27]. Here, 18 h exposure of HepG2 cells to IMT was enough to decrease cell viability when compared to the untreated control significantly. Recent studies of IMT effects

on HepG2 cells (24-72 h exposure) indicated that its antiproliferative effect is associated with a decreased expression of S-phase kinase-associated protein-2, a biomarker associated with a negative prognosis of cancers [25,26]. The up-regulation of non-metastatic protein 23, a kinase associated with metastasis when downregulated, was also shown to be part of the antiproliferative mechanisms of IMT on HepG2 cells [27]. A study combined IMT to dexketoprofen in liposomes against fibrosarcoma but was not as effective as a PEGylated nanocochleate formulation with the drugs [28].

SUT inhibits varied receptor tyrosine kinase and growth factor receptors involved in cancer progression [29,30]. In agreement with our data, an *in vitro* study described that the viability of HepG2 cells decreased to more than half of the initial value after 4 h exposure to SUT, and almost full inhibition cell growth was achieved after 24 h treatment at 5 mg/mL [29]. A phase II study with 34 HCC patients treated with 37.5 mg SUT (daily administrations, four weeks) indicated that although adverse effects were detected, antitumor activity was confirmed for advanced-stage HCC [30]. SUT liposomes tested in the present study could reduce HepG2 cells viability by nearly 80%. Unlike our data, a study indicated that SUT liposomes were modestly promoting pheochromocytomas growth suppression in an animal model with nude mice [31]. This might be partially explained not only by the cancer cell type, which may respond differently to antitumoral drugs but also by differences in the composition of the liposomes, which largely influences drug release.

BTZ inhibits the 26S proteasome from the ubiquitin-proteasome pathway, hampering tumorigenesis and uncontrolled cell division [32-36]. A tendency of better results was detected for BTZ through statistical analysis, following an 18 h treatment. A study in which HepG2 cells were treated with BTZ (48 h) described the downregulation of expression of E2F2 protein and up-regulation E2F6 protein, which present pro- and antiproliferative effects, respectively [32]. Exposure of HepG2 cells to BTZ (72 h) effectively decreased their viability and resulted in inhibition of the activity of the proteasome, chymotrypsin-like, and peptidylglutamyl-peptide hydrolyzing enzymes and promoted accumulation of Bax and Noxa proteins, which are associated with apoptosis [33]. BTZ liposomes were effective in containing the growth of multiple myeloma cell lines *in vitro* experiments and using an animal model of mice [34,35]. These drugs represent relevant possibilities for HCC treatment, considering the mechanisms of the disease [36-38].

4. Conclusions

Liposome entrapped IMT, SUT and BTZ were effective against the *in vitro* model of HCC in concentrations approximately 400-fold lower than their free counterparts. Although this is an *in vitro* study, our data are promising for better management of HCC, suggesting that liposomal preparations of these drugs might be important options for therapeutic success, especially BTZ.

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Conflicts of Interest

The authors declare no conflict of interest.

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