An Acad Bras Cienc (2023) 95(Suppl. 1): e20211078 DOI 10.1590/0001-3765202320211078
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

BIOMEDICAL SCIENCES

4-amino-2-phenyl-6-(*p*-fluorophenyl)-5-carbonitrile-pyrimidine-bis-substituted-loaded liposomes as promising system for cancer treatment

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Abstract: The aim of the present study was to perform *in vitro* and *in vivo* assessments of the antineoplastic action of 4-amino-pyrimidine encapsulated in liposomes. Liposomes were prepared and characterized for particle size and drug encapsulation and submitted to long-term stability tests. Cytotoxicity assays were performed in HeLa cells. Antineoplastic activity was investigated using the experimental sarcoma 180 tumor in Swiss albino mice. Encapsulation efficiency was 82.93 ± 0.04% and no significant changes were found with respect to particle size or pH after centrifugation and mechanical agitation tests. The *in vitro* results at concentration of 20 µg/mL indicated a considerable reduction in cell viability after treatment with encapsulated pyrimidine (75.91%). The *in vivo* assays using the compounds in encapsulated and free forms and 5-fluorouracil achieved tumor inhibition rates of 66.47 ± 26.8%, 50.46 ± 16.24% and 14.47 ± 9.22%, respectively. Mitotic counts demonstrated a greater reduction in the number of mitoses in animals treated with liposomal pyrimidine (32.15%) compared to those treated with the pyrimidine free (87.69%) and 5-fluorouracil (71.39%). This study demonstrated that the development of liposome formulations containing 4-amino-pyrimidine is a promising alternative for overcoming limitations related to the toxicity of current cancer treatment, ensuring greater therapeutic efficacy.

Key words: Antitumor activity, hela cells, liposomes, mitotic count, pyrimidine.

INTRODUCTION

Cancer comprises a group of diseases characterized by the uncontrolled multiplication of cells capable of developing and spreading tumors throughout the body (Pérez-Herrero & Fernández-Medarde 2015). According to the World Health Organization (WHO), cancer was the first or second leading cause of death before age 70 in 112 countries in 2019 and ranked third or fourth in 23 other countries (Sung et al. 2021). Despite considerable progress in cancer therapy, GLOBOCAN reports that around 19.3 million new cases of cancer occurred worldwide in 2020, with almost 10.0 million deaths and an expected increase to 28.4 million by 2040 (Sung et al. 2021).

Chemotherapy is the main form of treatment for most malignant tumors due to its ability to reach metastatic cells. However, this treatment modality has numerous side effects (Jesus et al. 2016). To find drugs that are effective at resisting anti-tumor cells and can reduce the occurrence of side effects caused by the use of

current anticancer drugs, new molecules lethal to neoplastic cells are under development, most of which have heterocycles in their structure, especially pyrimidines (Zarenezhad et al. 2021). The pyrimidine nucleus is one of the constituents of DNA and RNA chains in living beings (Jubeen et al. 2018) and is found in the scaffold of drugs widely used against diseases such as cancer, i.e., 5-fluorouracil (5-FU) (Vodenkova et al. 2020), and AIDS, i.e., zidovudine (de Clercq 2010). The pyrimidine nucleus has also been the object of numerous studies for the development new prototypes with several biological activities (de Coen et al. 2016), such as antibacterial (Verbitskiy et al. 2015), anticancer (Zarenezhad et al. 2021), antiviral (Zhuang et al. 2020) and antiparasitic (de Melo et al. 2018).

Despite considerable advances in the production of molecules for cancer therapy, directing the action to neoplastic cells is a line of research of considerable growth (Pérez-Herrero & Fernández-Medarde 2015) and has achieved success in overcoming harm to non-neoplastic cells. Such studies are based on the production of drug delivery systems (Dianat-Moghadam et al. 2018). Nano-encapsulation systems, such as liposomes, improve the solubility of substances, serving as carriers to the target, promote better stability of the compound within the body by interfering with the process of biodegradation or systemic biodistribution and favor the pharmacokinetics of therapeutic agents, which would drastically decrease the therapeutic index and, consequently, the toxic effects of drugs (Belfiore et al. 2018).

De Melo et al. (2002) developed pyrimidine prototypes using derivatives with numerous biological activities. The present study involves the encapsulation of 2-phenyl-4-amino-6-(*p*-fluorophenyl)-5-carbonitrile-pyrimidine (Pyr-Free) and the use of this encapsulated compound in studies with the HeLa cell line (cervical epithelial carcinoma) and sarcoma 180. Sarcoma-180 was discovered in 1914 as a solid mass in the right armpit of a white mouse (Qi & Xu 2006). The tumor invades skeletal muscle, fatty tissue, nerves and blood vessels. Despite its locally aggressive behavior, this neoplasm does not metastasize (Kurashige & Mitsuhashi 1982). Thus, the aim of the present study was to investigate the optimization of the antineoplastic potential of 4-amino-pyrimidinic-bis-substituted derivatives.

MATERIALS AND METHODS

Materials

The synthesized 4-amino pyrimidine compound was kindly supplied by the Research Group on New Bioactive Drugs and Natural Products supervised by Prof. Dr. Emerson Peter da Silva Falcão. Cholesterol (CHO), stearylamine (SA) and 5-FU were purchased from Sigma-Aldrich (St Louis, USA). Soybean phosphatidylcholine (PC) was obtained from Lipoid GmbH (Ludwigshafen, Germany). Methanol and chloroform were obtained from Merck (Darmstadt, Germany). Aqueous solutions were prepared with purified water obtained from the Human UP 900 purification system (Human Corporation, Seoul, Korea).

Synthesis of pyrimidine compounds

The pyrimidine derivative was produced by the Research Group on New Drugs and Bioactive Natural Products through consecutive reactions (Figure 1) using the method described by de Melo et al. (2002). An appropriate aromatic aldehyde initially (Figure 1a) reacted with malononitrile (Figure 1b) to give substituted bisnitrile (Figure 1c), which was purified and crystallized and then reacted with an amidine (Figure 1d), yielding the compound Pyr-Free (Figure 1e). The synthetic reaction was monitored by thin

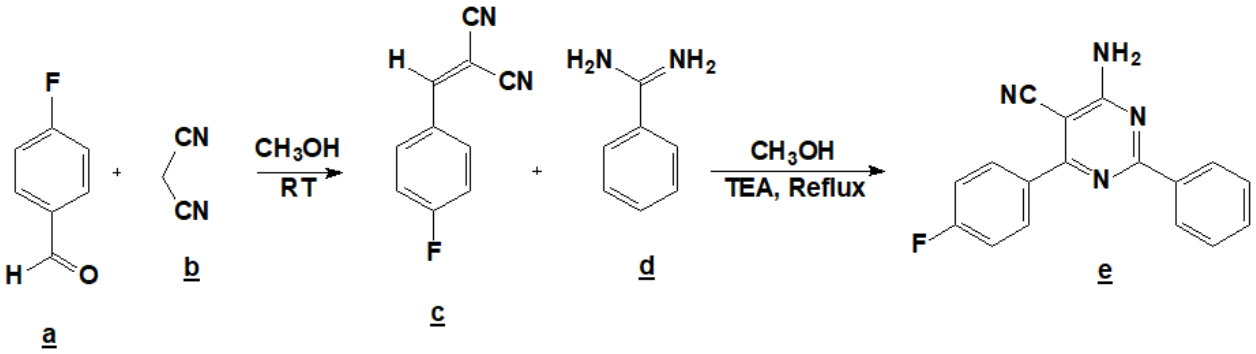

Figure 1. **Reaction scheme. a: Aromatic aldehyde; b: Malononitrile; c: Substituted bisnitrile; d: Amidine; e: 2–Phenyl–4–amine–5–carbonitrile–6–(*p*–fluorophenyl)–pyrimidine.**

layer chromatography (TLC). The synthetic structure was confirmed by the common technique of mass spectrometry (Delsi-Nermag mass spectrometer coupled to a HP 5890 gas chromatograph), Nuclear Magnetic Resonance (NMR) (UNITY spectrophotometer, Varian model), infrared spectrometry (Burker spectrometer, IFS 66 model) and the melting point (Electrothermal digital - model 9100).

Preparation of liposomes containing 2-phenyl-4-amino-6-(*p*-fluorophenyl)-5 carbonitrile-pyrimidine compound

Liposomes containing the 4-amino-pyrimidine-bis-substituted compound (Pyr-LIPO) were prepared using the hydration lipid film method (Lira et al. 2009). Lipids (PC, CHO and SA - 7:2:1, 42 mM) and the pyrimidine derivative (10 mg) were solubilized in a mixture of chloroform and methanol (3:1 v/v). The mixture was submitted to vacuum evaporation for complete elimination of the organic solvents and consequent formation of a thin lipid film. The film was then hydrated with 10 mL of 7.4 pH phosphate buffer to form Multilamellar Lipid Vesicles (MLVs). Small Unilamellar Vesicles (SUVs) were obtained from the sonication of the MLVs (Vibra cell, BRANSON, USA) at 200 W and 40 Hz for 300 s.

Characterization of liposomes containing 2-phenyl-4-amino-6-(*p*-fluorophenyl)-5-carbonitrile-pyrimidine compound

The following physicochemical characteristics of the Pyr-LIPOs were analyzed: macroscopic appearance, pH, particle size, polydispersity index (PDI), zeta potential and drug encapsulation efficiency. Pyr-LIPOs were checked at predetermined times to determine stability.

In the study of physical stability under forced conditions, the preparations were submitted to stress conditions to simulate processes such as those occurring during transport and storage (Lapenda et al. 2012). For such, the formulations were aliquoted and subjected to centrifugation at 6000 rpm for 1 h at 4 ± 1 °C (KN-70 centrifuge, Kubota, Japan), simulating the accelerated passage of time. For the mechanical stress tests, the samples were placed into an microtube, immersed in a water bath and subjected to horizontal shaking at 180 vibrations/min at a controlled temperature of 37 ± 1 °C for 48 h (Polytest® 20 Bioblock Scientific, France) to simulate transport conditions. Stability tests were conducted at predetermined times (0, 7, 15 and 30 days) (Santos-Magalhães et al. 2000) and the formulations were maintained at 4 °C and under atmospheric pressure.

Macroscopic analysis was performed to observe changes in the general appearance of the preparations, such as homogeneity, color, viscosity, deposition of materials, formation of crumbs, cremating, flocculation, coalescence and separation of phases. Particle size and the polydispersity index were determined by photon correlation spectroscopy using a laser particle size analyzer (Beckman Coulter, UK). For such, three samples of each liposome (50 µL) formulation were diluted in ultra-pure water (950 µL) (Lapenda et al. 2012). The zeta potential (surface charge of vesicles) was measured by determining electrophoretic mobility (Nanotrac®, USA). Three 50-µL samples were diluted in 950 µL of ultrapure water. All results were expressed as mean ± standard deviation (SD).

Drug content and encapsulation efficiency were analyzed by UV-Vis spectrophotometry (Ultrospec® 3000 pro, Amersham Biosciences, Sweden). Analyses were performed in triplicate and values were expressed as mean ± SD. The content of the 4-amino-pyrimidine-bis-substituted compound was quantified by withdrawing an aliquot of the liposome suspension (20 µL), which was dissolved in methanol and sonicated for 5 min. The compound was then quantified by spectrophotometry (260 nm) using the standard 4-amino-pyrimidine-bis-substituted curve with concentrations ranging from 0.5 to 5 µg/mL in methanol.

Encapsulation efficiency was determined by the ultrafiltration/ultracentrifugation technique (Andrade et al. 2004, da Silva Santos et al. 2006) using Microcon® units (Millipore, USA). An aliquot of the liposome formulation (400 µL) was centrifuged at 10,000 rpm for 1 h at 4 °C. The filtrate was quantified by UV-Vis spectrophotometry (260 nm). Encapsulation efficiency was calculated by the equation EE% = ((total 4-amino-pyrimidine-bis-substituted compound) – (4-amino-pyrimidine-bis-substituted free) / (total of 4-amino-pyrimidine-bis-substituted compound)) × 100%.

Cell culture

In vitro assays were performed at the NanoBioCel Laboratory (UFPE – CAV) using the HeLa cell line in the exponential growth phase. The cells were obtained from the Cell Bank of Rio de Janeiro (BCRJ), cultured in a DMEM culture medium supplemented with 10% fetal bovine serum, 1% antibiotic solution (penicillin 1000 IU/mL + streptomycin 250 mg/mL) and 1% L-glutamine 200 mM and incubated at 37 °C in a 5% CO2 atmosphere with 80% humidity.

Determination of cell viability

Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann 1983), which is based on the reduction of the yellow salts of tetrazolium by mitochondrial reductase from metabolically active cells. Purple crystals are formed intracellularly, which are solubilized and further analyzed by UV-visible spectrophotometry. A lower MTT reduction and lower spectrophotometric signal indicate lower cell viability.

The cell suspension (10^5 cells/mL) was distributed in a 96-well culture plate (198 µL/well). The plates were incubated at 37 °C in an incubator (Sedas, Milan-Italy) with a humid atmosphere enriched with 5% CO_2. After 24 h of incubation, the 4-aminopyrimidine-bis-substituted compound in its free form (DMSO-diluted) and encapsulated forms, in addition to 5-FU, were diluted in DMEM to obtain final concentrations of 20, 10, 5 and 2.5 µg/mL. Next, 22 µL of each solution were added to the wells. Untreated cells were used as control. The cytotoxic effects of the test samples were assessed after 72 h and the cells were incubated for 3 h with MTT for further reading. Absorbance

was read using a plate reader at a wavelength of 490 nm. Mean absorbance of the control group was considered 100% viability. Thus, the results were assessed by determining the inhibition of cell proliferation in relation to the control. Each test was performed three times. In the analyses, the results were expressed as a mean ± SD and were interpreted using analysis of variance (ANOVA) with Bonferroni's correction to identify significant differences ($p < 0.05$). Statistical analysis was performed with the aid of the GraphPad Prism 5.0 program.

In vivo antitumor assay

Experimental animals

Male Swiss albino (Mus musculus) mice aged 35 to 60 days with a mean weight of 30.0 g (n = 24) were acquired from the Keizo Asami Immunopathology Laboratory (UFPE) and kept at 22 °C ± 2 °C under a natural 12-h light/dark photoperiod with free access to water and food throughout the experiment. The animals were distributed in groups according to experimental design (negative control group treated with saline, positive control groups treated with 5-FU, Pyr-free and Pyr-LIPO). The experiments were conducted in accordance with the protocol of the UFPE Ethics Commission on Animal Experimentation (Recife, Brazil) (process no. 23076.050686/2012-43).

Antitumor activity of 4-amino-pyrimidine-bis-substituted compound in free and encapsulated forms

The *in vivo* antitumor activity of the 4-amino-pyrimidine-bis-substituted compound was assessed in its free and encapsulated forms against sarcoma 180. Tumor cells (5.0×10^6 mL^{-1} cells) in ascitic form were obtained by punction and subcutaneous inoculation in the right axillary region of the mice. The test was performed with four groups of six animals each (negative control group treated with saline, positive control group treated with 5-FU, Pyr-free and Pyr-LIPO). Treatment began 24 h after tumor inoculation and was carried out for seven consecutive days. Injections of free 4-amino-pyrimidine-bis-substituted solutions and encapsulated in liposomes at a dose equivalent to 15 mg/Kg of body weight, 5-FU (positive control) at a dose of 20 mg/Kg and weight-dependent placebos (saline) were given intraperitoneally. The therapeutic doses were studied previously (Da Silva et al. 2008, Falcão et al. 2006) The 4-amino-pyrimidine-bis-substituted compound was dissolved in a solution of 2.5% Tween 80 in sterile 0.9% NaCl solution.

After one week of treatment, the animals were sacrificed with an overdose of urethane (1.25 g/kg). The tumors were removed and weighed prior to microscopic analysis. Tumor inhibition was determined from the mean tumor weight of the groups of treated animals relative to the untreated control group using the following equation: IT% = (C-T / C) × 100%, in which C is the mean tumor weight of the animals from the control group (saline solution) and T corresponds to the mean tumor weight of the animals in the treated groups (Pyr-free, Pyr-LIPO and 5-FU).

Mitotic counts

The tumors were removed and submitted to mitotic count analysis. The tissues were fixed in 10% buffered formalin and embedded in paraffin. Sections were cut using a microtome set to 4 µm and submitted to hematoxylin-eosin staining. The histological images of the slides were captured by a digital camera (Moticam 3.0) coupled to the optical microscope (Nikon-E200) with a fixed focus and field clarity, obtaining 10 fields per slide with a final magnification of 400 x. The photomicrographs were evaluated using

ImageJ version 1.44 (Research Services Branch, US National Institutes of Health, Bethesda, MD, USA). In each field, the number of mitoses were counted and determined as the mean number of mitoses per tumor. To create the graph, the values were normalized to the control, which was considered equal to 100%. The data from the histomorphometric analysis were statistically analyzed. The results were expressed as a mean ± SD and were interpreted using ANOVA with Tukey's post hoc test to identify significant differences ($p < 0.05$). Statistical analysis was performed with the aid of the GraphPad Prism 5.0 program.

RESULTS AND DISCUSSION

Preparation and characterization of liposomes

Pyr-LIPO (1 mg/mL) was prepared using the lipid film hydration method, which is suitable for the encapsulation of liposoluble substances, as the low polarity makes these substances insoluble in aqueous solutions (identified by solubility tests). The organic phase (chloroform:methanol) was added and the compound could then be incorporated into the liposome membranes (Frézard et al. 2005). After the preparation process, the liposome formulations had an opaque, homogeneous milky appearance with an evident Tyndall effect, confirming the presence of SUVs (Lapenda et al. 2012). A stability study was performed to analyze the behavior of the 4-amino-pyrimidine-bis-substituted compound in the formulations. A previous study demonstrated an interesting interaction between 4-amino pyrimidine compounds and lipid membranes (Luna et al. 2011), avoiding possible drug precipitation. Macroscopic aspects, particle size, PDI and pH of the formulations were analyzed in the study of physical stability in forced conditions (Table I), as recommended in the literature (Batista et al. 2007). The liposome formulations containing the 4-amino-pyrimidine-bis-substituted compound did not present changes in visual appearance

Table I. Study of physical stability in forced conditions of liposomes containing 4-amino-pyrimidine-bis-substituted compound.

PARAMETERS	Unloaded-LIPO	Pyr-LIPO
INITIALS	Ø: 138.45 ± 25.04 PDI: 0.32 ± 0.04 pH: 6.53 ± 0.05 macroscopic evaluation: no changes	Ø: 270.04 ± 24.94 PDI: 0.39 ± 0.10 pH: 7.17 ± 0.23 macroscopic evaluation: no changes
CENTRIFUGATION	Ø: 149.55 ± 9.83 PDI: 0.31 ± 0.05 pH: 7.34 ± 0.13 macroscopic evaluation: no changes	Ø: 276.90 ± 35.21 PDI: 0.34 ± 0.05 pH: 7.16 ± 0.16 macroscopic evaluation: no changes
MECANIC STRESS	Ø: 103.82 ± 8.73 PDI: 0.36 ± 0.01 pH: 6.58 ± 0.10 macroscopic evaluation: no changes	Ø: 285.18 ± 20.88 PDI: 0.38 ± 0.02 pH: 7.41 ± 0.13 macroscopic evaluation: no changes

Ø: Particle size; PDI: Polydispersity Index; pH: Hydrogenic potential; Micro and macroscopic analysis: homogeneity, color, viscosity, deposition of material, formation of lumps, flocculation, coalescence and separation of phases.

and were stable during centrifugation and mechanical stress, as shown in Table I.

Stability tests at predetermined times (Table II) performed over a 30-day period demonstrated no macroscopic changes and the formulations maintained the characteristics presented after preparation. The diameter of the liposomes containing 4-amino-pyrimidine-bis-substituted ranged from 270.04 ± 24.94 (Day 0) to 341 ± 25.32 (Day 30), with PDI values around 0.37. The diameter increased due to the presence of the lipolytic substance (Chorilli et al. 2007). Moreover, the discrete increase may be related to the tendency of the particles to aggregate over time (Schaffazick et al. 2003). No significant changes were found in the PDI, indicating homogeneity in the distribution of vesicle size (Chorilli et al. 2007). A slight decrease in pH was found between the onset of the test (7.17 ± 0.23) and the last day of assessment (6.61 ± 0.12). However, these variations do not constitute instability in the formulations.

The pyrimidine-loaded liposomes had a zeta potential value of 37.7 ± 2.26, whereas the unloaded liposomes had a potential of 37.5 ± 1.62. The presence of positively charged lipid stearylamines in the bilayer composition gives liposomes a positively charged surface. The similar zeta potentials of the pyrimidine-loaded and unloaded liposomes indicate no drug on the surface of the liposomes, remaining in the lipid bilayer, as expected. Liposomes with negative or positive zeta potential values above 30 mV are stable in suspensions, as repulsive forces prevent the aggregation of the vesicles (Calvo et al. 1997, Schaffazick et al. 2003). Divergent opinions are found in the literature regarding the interaction between the charge of the nanocarrier and tumor. However, Honary & Zahir (2013) found that nanoparticles with a positive charge are preferentially absorbed by tumor cells and retained for a longer time compared to neutral or negative nanoparticles. The authors also found that a more positive nanoparticle surface led to stronger bonds between the nanoparticle and plasma membrane of tumor cells, which are generally anionic. Thus, the positive zeta potential values likely determine that the liposomes developed in this investigation contribute to the increase in the antitumor activity of the pyrimidine compound.

Encapsulation efficiency calculated by relating the total and free percentage of the compound was 82.93 ± 0.04. The standard curve equation was Y= 0.1675X + 0.0442, in which the X axis is the concentration of liposome (dissolved in analytical grade methanol) and the Y axis

Table II. Study of stability at predetermined times (0 to 30 days) of blank liposomes and liposomes containing 4-amino-pyrimidine-bis-substituted compound.

	Unloaded LIPO			Pyr-LIPO		
(days)	**Ø**	**PDI**	**pH**	**Ø**	**PDI**	**pH**
0	138.45 ± 25.04	0.32 ± 0.04	6.53 ± 0.05	270.04 ± 24.94	0.39 ± 0.10	7.17 ± 0.23
7	151.86 ± 26.31	0.26 ± 0.03	6.22 ± 0.02	287.88 ± 20.58	0.37 ± 0.01	7.12 ± 0.39
15	151 ± 27.47	0.31 ± 0.18	6.01 ± 0.11	295.74 ± 73.10	0.36 ± 0.03	6.58 ± 0.04
30	170 ± 12.94	0.33 ± 0.05	6.02 ± 1.33	341.35 ± 25.32	0.36 ± 0.02	6.61 ± 0.12

Formulations maintained at 4 °C and under atmospheric pressure. Ø: Particle size; PDI: Polydispersity Index; pH: Hydrogenic potential.

corresponds to absorbance at the wavelength of 203 nm, with an R^2 = 0.9885.

Cell viability study

The viability study with the HeLa cell line was performed to assess the effect of the 4-amino-pyrimidine-bis-substituted compound in its free and encapsulated forms and 5-FU, which is a drug with known activity. Figure 2 demonstrates the cell viability of HeLa versus the compounds tested. An increase in the concentration of the compounds generated a decrease in cell viability, denoting increased mortality, as reported in the literature (Moghimipour et al. 2018). Pyr-LIPO exhibited greater cytotoxicity compared to the other compounds tested at concentrations of 5, 10 and 20 µg/mL. This result is in agreement with data described in the literature (Mirgorodskaya et al. 2020), showing an increase in the therapeutic efficacy of compounds when encapsulated.

Cytotoxic activity of the compounds was determined according to the National Cancer Institute (NCI) scale (Cocco et al. 2006). Compounds are considered to lack cytotoxic activity when inhibition is in the range of 1-20% and exhibit little activity when between 20 and 50%, moderate activity when inhibition is 50 to 70% and high activity when the inhibition range is between 70 to 100%. Therefore, the compound encapsulated at the concentration of 20 µg/mL had high activity (75.91%), while the free compound at the same concentration had little activity (46.63%) and 5-FU had moderate activity (60%). These data underscore the advantages of liposomes and are in agreement with the results of the analyses carried out in this study, demonstrating that the compound encapsulated in liposomes has greater cytotoxicity at lower concentrations compared to the other compounds tested, even 5-FU.

Study of antitumor activity *in vivo*

In addition to the 4-amino-pyrimidine-bis-substituted compound in its free and encapsulated forms, we used 5-FU as a positive control in this study because this drug has been shown to be clinically effective against cancer in mice and humans (Zhang et al. 2012) and is a pyrimidine analog, thus a possible mechanism of action may be attributed to the drug. The doses used were defined based on preliminary studies conducted by Falcão et al. (2006) with the aim of defining the toxicity of some pyrimidine derivatives, demonstrating that these derivatives have low toxicity. Thus, a minimal dose capable of producing an antitumor effect was defined

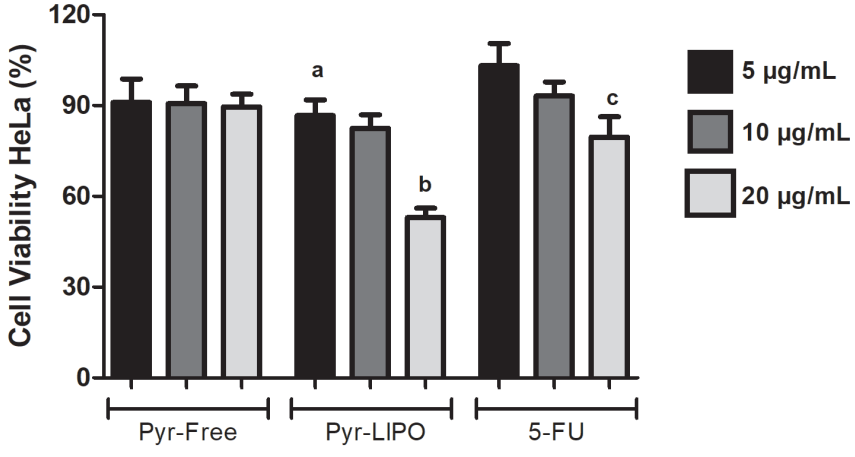

Figure 2. Viability of HeLa cells exposed to free pyrimidine (Pyr-Free), encapsulated pyrimidine (Pyr-LIPO) and 5-fluororucil (5-FU). Statistically significant differences between groups determined by ANOVA with Bonferroni correction, *p < 0.05. Data expressed as mean ± SD of three independent experiments. "a" significantly less than 5-FU at same concentration; "b" significantly less than Pyr-Free and 5-FU at same concentration; "c" significantly less than Pyr-Free.

for this study. Antitumor activity was calculated by comparing the mean weight of the tumors from the various experimental groups to that of the control group. After treatment, a change was found in tumor weight (Figure 3). A greater reduction was found in the group treated with liposomes containing the 4-amino-pyrimidine-bis-substituted compound (1.42 ± 1.14 g), compared to its free form (2.10 ± 0.69 g) and 5-FU (3.63 ± 0.39 g). The animals in the control group had tumors with a mean mass of 4.24 ± 0.91 g. These results demonstrate that Pyr-LIPO produced a greater regressive effect on tumor mass than Pyr-Free and even 5-FU, which has recognized antitumor activity.

The animals treated with Pyr-LIPO presented satisfactory tumor inhibition of 66.47 ± 26.8% (Figure 4). According to the NCI, tumor inhibition rates above 60% are considered satisfactory. The suspension compound achieved 50.46 ± 16.24% inhibition, whereas inhibition in the 5-FU group was 14.47 ± 9.22%, which was less than that produced by the compound tested. The antitumor activity of compounds containing the pyrimidine scaffold has been described in the literature and is attributed to the presence of the pyrimidine ring and *p*-fluorophenyl region (Muthuraja et al. 2019).

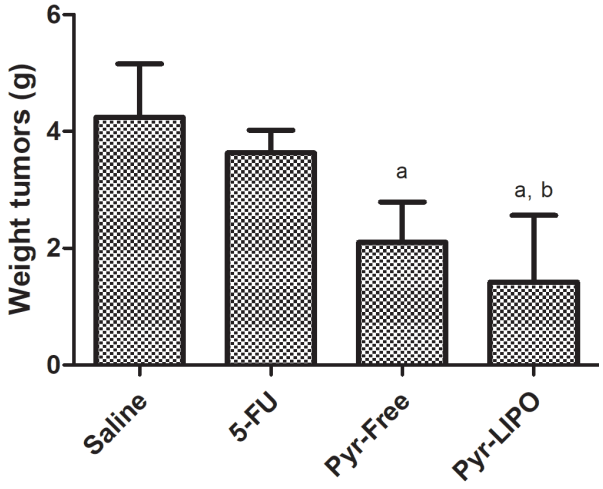

Figure 3. Tumor weight in groups treated with saline solution, 20 mg/kg body weight of 5-fluorourayl (5-FU) and 15 mg/kg body weight of free (Pyr-Free) and encapsulated 4-amino-pyrimidine-bis-substituted compound (Pyr-LIPO). Statistically significant differences between groups determined by ANOVA followed by Tukey's post hoc test, *p < 0.05. Data expressed as mean ± SD of three independent experiments. "a" significant difference compared to saline group; "b" significant difference compared to 5-FU group.

The increase in the effectiveness of these compounds when encapsulated, however, has not previously been reported in the literature, although some studies report that liposomes are able to increase the effectiveness of the substances they encapsulate. Pyrimidine derivatives, such as CDK inhibitors, α-TNF inhibitors, protein tyrosine kinase inhibitors, kinase inhibitors (PI-3 and AKT) and cytokine inhibitors, have potent antitumor activity (Xie et al. 2009). 5-FU, which is a pyrimidine analogue, mainly interferes with thymidylate synthesis (Zhang et al. 2012). It is possible that the 4-amino-pyrimidine compound studied has a similar mechanism of action, but additional data are needed to infer the specific mechanism with certainty.

The increased inhibition found with the 4-amino-pyrimidine compound when encapsulated demonstrates that liposomes may be suitable carriers for this class of compounds used for antitumor therapy due to the ability to enhance drug effectiveness (Pérez-Herrero & Fernández-Medarde 2015). It is possible that this effect is due to a change in the pharmacokinetics and biodistribution of the drug, as documented for other antineoplastic drugs (Dianat-Moghadam et al. 2018).

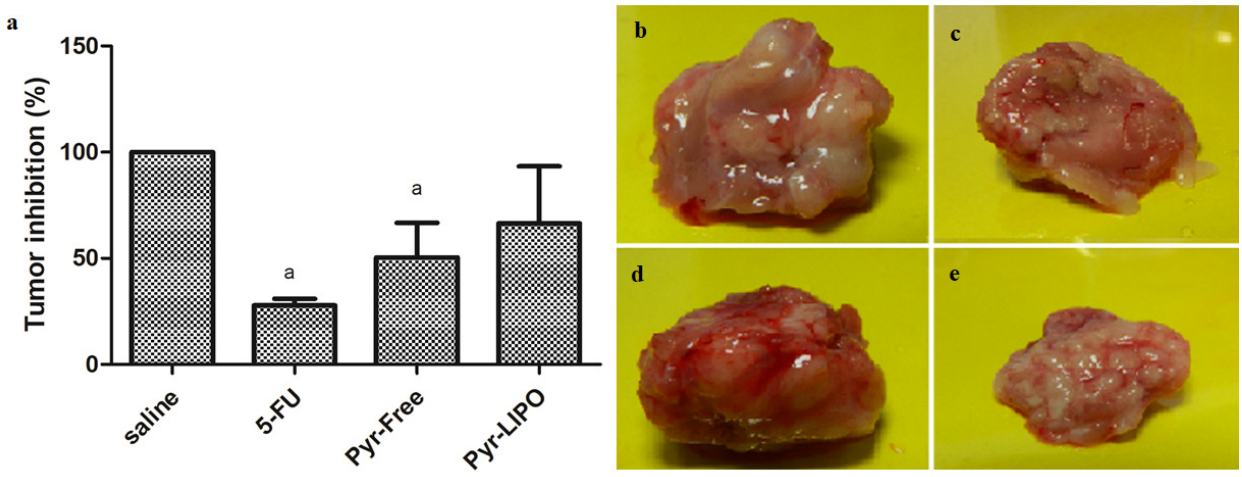

Figure 4. Tumor inhibition in groups treated with free 5-FU (20 mg/kg), Pyr-Free (15 mg/kg body weight) and Pyr-LIPO (15 mg/kg of body weight) (a). Macroscopic aspect of tumor after treatment with saline (b), 5-FU (c), Pyr-Free (d) and Pyr-LIPO (e). Statistically significant differences between groups determined by ANOVA followed by Tukey's test, *p < 0.05. Data expressed as mean ± SD of three independent experiments. "a" significant difference compared to saline group.

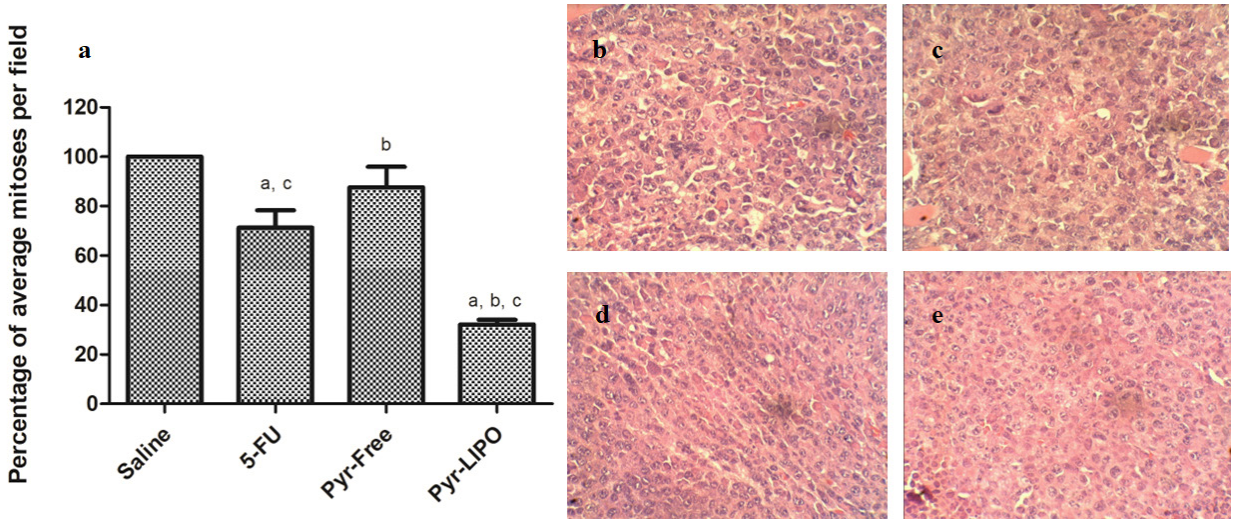

Figure 5. Effect of administration of free 5-FU (20 mg/kg), Pyr-Free (15 mg/kg body weight) and Pyr-LIPO (15 mg/kg body weight) on quantity of mitoses. Values normalized to control (100%) (a). Histological photomicrographic images stained with hematoxylin/eosin of groups treated with saline (b), 5-FU (c), Pyr-Free (d) and Pyr-LIPO (e). * Indicates statistically significant differences (p < 0.05). Significant differences between groups determined by ANOVA followed by Tukey's test, *p < 0.05. Data expressed as mean ± SD of three independent experiments. "a" significant difference compared to saline group; "b" significant difference compared to 5-FU group; "c" significant difference compared to Pyr-Free group.

Determination of mitotic count

Regardless of the group studied, the tumors invaded muscle, bone and fatty tissues. Areas of necrosis and hemorrhage were also found. Neoplastic cells were predominantly arranged in a large solid pleomorphic pattern, with abundant cytoplasm and no defined edges. The nuclei were hyperchromatic and large and ranged in shape from oval to elongated. Several mitotic figures were observed, some of which were atypical. As the number of mitoses expresses cell division activity, a greater number

of mitoses denotes greater proliferative activity in the tissue (Ministério da Saúde 2019). Thus, the mitotic count is an important indicator of adequate cell proliferation (Gadano et al. 2002), enabling better knowledge on mitoses (degree of cellular activity of the tissue under study) and providing information on biological behavior. The inhibition of cell division demonstrated by the mitotic index values (Figure 5) shows greater inhibition of the number of mitoses in animals treated with the Pyr-LIPO compound (32.15%) compared to those treated with 5-FU (71.39%) and Pyr-Free (87.69%). This demonstrates that the compound tested has an antiproliferative capacity that is enhanced by encapsulation in liposomes, thereby increasing the therapeutic efficacy of the compound.

The results demonstrated that liposome formulations containing the compound 2-phenyl-4-amino-6-*p*-fluorophenyl-5-carbonitrile-pyrimidine increased the effect and efficacy of the test compound compared to use in its free form, producing greater tumor inhibition compared to the control group and 5-fluoracil. Therefore, the development of liposomes containing the 4-amino-pyrimidine-bis-substituted compound is an option that can be adopted to overcome limitations related to current antineoplastic treatment, thus ensuring safety and therapeutic efficacy. Furthermore, the compound was shown to be more effective than 5-FU, which is a clinically accepted drug in antitumor therapy.

Acknowledgments

The authors are grateful to the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the financial assistance granted through a Master's scholarship provided through the Programa de Apoio a Planos de Reestruturação e Expansão das Universidades Federais (REUNI).

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How to cite

OLIVEIRA JV, ALMEIDA GC, NOGUEIRA MCBL, AGUIAR JÚNIOR FCA, MELO AO, SILVA TDS, SANTOS NPS, MAGALHÃES NSS, MELO SJ & FALCÃO EPS. 2023. 4-amino-2-phenyl-6-(*p*-fluorophenyl)-5-carbonitrile-pyrimidine-bis-substituted-loaded liposomes as promising system for cancer treatment. An Acad Bras Cienc 95: e20211078. DOI: 10.1590/0001-3765202320211078.

Manuscript received on May 13, 2021;
accepted for publication on November 8, 2022

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