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IN VITRO COMPARATIVE STUDY OF THE ANTITUMORAL EFFECTS OF AMPHOTERICIN B AND ITS DERIVATIVE AMPHOTERICIN A21 IN LUNG CELLS

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ABSTRACT
The complications that occur in cancer treatment require therapies that, in addition to increased effectiveness, can also reduce side effects. Amphotericin-A21 (AmB-A21) is a promising amphotericin B derivative that has the same antifungal effect but has been shown to be less toxic than its precursor. The present study investigates whether AmB-A21 has antineoplastic effects and whether these are potentiated when combined with carboplatin. To evaluate antineoplastic capacity, we performed curve dose-response of AmB-A21, AmB, and carboplatin. Then, A549 cells were treated with the corresponding IC_{25} of each drug: 67 µg/ml AmB-A21, 29 µg/ml AmB, and 515 µg/ml carboplatin, alone or combined during 24 h. We evaluated effects on viability, proliferation, apoptosis, and genotoxicity. Our result demonstrated that AmB-A21 produced cytotoxicity, inhibited cell proliferation, induced apoptosis, And induced genotoxicity of A549 cells. AmB-A21 was more effective in reducing cell proliferation than AmB. The combination of AmB-A21 with carboplatin had a more pronounced effect. Based on these results, we consider AmB-A21 to be a potential candidate for antitumor treatment.

KEYWORDS: Amphotericin A21, Amphotericin B, Cytotoxicity, Apoptosis, Proliferation, invasiveness, Carboplatin.

INTRODUCTION
Estimates from recent years indicate that lung cancer is the second most diagnosed type of cancer and the first cause of death from cancer worldwide.[1] Lung cancer diagnosis is usually made during advanced stages of the disease, so treatment is not carried out in a timely manner. This type of cancer is characterized by a poor level of response to treatment and a tendency to develop resistance to it.[2,3]

One of the most widely used tools for the treatment of this type of cancer is chemotherapy. First choice drugs are platinum derivatives, such as cisplatin and carboplatin.[4,5] Both antineoplastic drugs have been shown to be effective in the treatment of lung cancer, alone or in combination with other therapies.[6] However, they cause adverse effects such as nephrotoxicity, hepatotoxicity, cardiotoxicity, myelosuppression and hypersensitivity.[7,8] Although there are various therapies and antineoplastic regimens, these still do not counteract the complications faced by patients and physicians. Hence, several research groups are seeking new drugs with antineoplastic properties and fewer serious side effects.[9]

One of the drugs recently studied for this purpose is amphotericin B (AmB). It has been found that AmB reduces cell proliferation, migration, and invasion, while also increasing apoptosis and cytotoxicity *in vitro*.[10-12] In addition, when in combination with conventional antineoplastic clinical drugs, it has enhanced antineoplastic effects.[13-16] However, its use is not ideal given its severe adverse effects, which would be added to the effects produced by antineoplastic therapy.[17-19]

Amphotericin A21 (AmB-A21, L-histidine methyl ester of amphotericin B), is a derivative of AmB that has been extensively studied on *in vitro* and *in vivo* models,[20] where it has been shown to be as effective as AmB against *Candida albicans* strains and in a candidiasis model.[21] Previous safety studies in animal models have shown that AmB-A21 does not produce the adverse effects of its precursor[22] and, as has also been noted, it induces cytotoxicity and apoptosis in tumour cells.[20] Therefore, the purpose of this study was to investigate whether amphotericin A21 has the same anti-tumor properties as its precursor, and whether it might expand and improve therapeutic options for cancer patients.

MATERIALS AND METHOD

A549 cell culture

A549 cells (ATCC CCL-185) were used to perform the experiments. Cell growth was undertaken with Kaighn's F-12 Ham modified medium (F-12K), purchased from SIGMA-ALDRICH®, supplemented with 10% fetal bovine serum (FBS) (GIBCO®). A549 cells were grown at 37 °C, 5% CO_2 and 95% air (culture conditions). Amphotericin (AmB), Amphotericin A21 (AmB-A21) and Carboplatin (CBP), were used as individual treatments. For the combined treatments, carboplatin was mixed with AmB and AmB-21. Prior to the experiments, concentration-response curves were performed to obtain inhibitory concentrations 25 and 50 (IC_{25}, IC_{50}) (Figure 1). Therefore, the concentrations used in all experiments for each drug were as follows: 29 µg/ml AmB, 67 µg/ml AmB-A21 y 515 µg/ml carboplatin.

Evaluation of Cytotoxicity and Cell proliferation in A549 cells

The 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction technique (MTT; Sigma-Aldrich®) was performed to evaluate the cytotoxicity produced by the different treatments.[23] In a 96-well plate (Corning Incorporated Life Science), 18,000 cells were seeded per well and incubated for 24h under the culture conditions. Subsequently, the cells were treated with the concentrations indicated above, during 24 h. A negative control group was included. Once the treatment time had elapsed, the medium was removed and replaced by un-supplemented medium. MTT (5mg/ml) reagent was subsequently added and incubated during 4h under culture conditions. After the medium was removed, Dimethyl sulfoxide (DMSO) with a purity of 99.9%, and Sorensen's buffer (0.1M glycine + 0.1M NaCl, at pH10.5) were added. Finally, the plate was read in a plate reader (Multimode Plate Reader VICTOR X3 PerkinElmer) at a wavelength of 550nm. It should be noted that, to obtain the inhibitory concentrations, dose response curves were performed for each compound prior to this experiment (Figure 1 shows the values of each IC_{25} and IC_{50}).

The MTT technique was also performed as previously described to evaluate the effect of the treatments on cell proliferation. For this experiment, 1,500 cells were seeded per well, and the steps described above carried out. Cells were treated with 29 µg/mL AmB, 67 µg/ml AmB-A21, 10µg/ml cisplatin y 515 µg/ml carboplatin, in single and combined treatments. The readings were made at 24, 48, 72 and 96 h of treatment, using their respective IC_{25}.

Identification of the cell death

The identification of the type of cell death produced by the treatments was carried out by staining with ethidium bromide plus acridine orange. A sterile coverslip was placed in 6-well plates (Corning Incorporated Life Science), and 250,000 cells were seeded per well and incubated for 24 h. Subsequently, cells were treated with 29 µg/ml AmB, 67 µg/ml AmB-A21, 10µg/ml cisplatin y 515 µg/ml carboplatin (alone or combined) during 24 h. A non-treated control was added. At the end of the treatment period, the coverslip was carefully removed and washed (with PBS). A mixture of ethidium bromide/acridine orange was placed on a slide (with a final concentration of 100 µg/ml of each reagent). The fixed cells were covered with another coverslip, and subsequently observed under inverted fluorescence microscopy (Olympus IX81). Representative microphotographs of each treatment were taken for analysis and quantification. Fiji software (ImageJ®) was used for the quantitative analysis of the images.

DNA damage evaluation

Assessment of DNA damage was performed with a commercial kit purchased from Invitrogen™ (HCS DNA Damage Kit, catalog number: H10292). The kit makes it possible to observe cells with DNA damage via red fluorescence, and nuclei (in living and dead cells) with blue fluoresce. Using 6-well plates, a sterile coverslip was placed in each well and 250,000 cells were seeded per well and incubated for 24 h, under culture conditions. Subsequently, cells were treated with 29 µg/ml AmB, 67 µg/ml AmB-A21, 10µg/ml cisplatin y 515 µg/ml carboplatin (alone or combined) during 24 h. A DNA damage positive control group (30% H_2O_2) was added. At the end of the treatment time, the test was performed on the coverslip, following manufacturer's protocol. Briefly, component A of the kit was added to the cells, and these were incubated for 30 min under culture conditions. Subsequently, the cells were fixed with 3.7% formaldehyde and subsequently permeabilized with 0.25% Triton® X100. The cells were then blocked with bovine serum albumin (BSA, 10 µg/ml) during one hour at room temperature. The BSA was then removed and incubated during 1h with the primary antibody pH2AX, at room temperature. At the end of the incubation, the primary antibody was removed, the cells were washed 3 times with PBS1X and incubated for 1h with the secondary antibody conjugated with Alexa Fluor 555 and Hoechst 33342 pH2AX, at room temperature and protected from light. The secondary antibody was removed, washed 3 times with PBS1X. Finally, treated cells were observed under a confocal microscope and representative images were obtained. The quantitative

analysis was performed on the images using Fiji software (ImageJ®).

Statistical analysis

The data were statistically analyzed using the IBM SPSS Statistics 25 software (SPSS Inc., Chicago, ILL., USA) and were represented as the mean ± standard deviation. The results were subjected to a one-way analysis of variance (ANOVA) and a Tuckey test. If the p value was less than 0.05, differences were considered statistically significant.

RESULTS

Concentration response curves were performed to obtain the IC_{25} for each drug and these were used for the individual and combined treatments in all experiments. The IC_{25} obtained for each drug are shown at the top of Fig. 1. As per our observations, AmB-A21, like AmB and carboplatin, was cytotoxic for A549 cells; the concentration at which cell viability was reduced by 25% (IC_{25}) was 67 µg/ml. The IC_{25} was about 2.3-fold more than AmB and 7.6-fold lower than carboplatin (Fig. 1). Regarding the combined treatments, it was observed that carboplatin + AmB-A21 treatment, reduced cell viability by 44% ($p<0.05$), as compared with the control group. The combined treatments that produced the least cytotoxic effect were carboplatin + AmB (35%) ($p<0.05$).

Fig. 2 shows the proliferation curves obtained from the individual and combined treatments. As can be seen, AmB-A21 inhibited proliferation 100% during the first 48 h ($p <0.05$). Later, inhibition was 60 and 70% at 72 and 96 h respectively ($p<0.05$). AmB inhibited cell proliferation by 90% at 24 h ($p <0.05$), and there was subsequently a reduction in the number of cells, probably due to cell death. There was, however, a tendency toward increased proliferation at 72 and 96 h ($p <0.05$). Carboplatin inhibited proliferation by 30% at 24h, ($p <0.05$). However, after 48 h, it began to reduce cell proliferation, probably due to cell death ($p <0.05$). All treatments combined showed a complete inhibition of proliferation with a significant reduction in cells ($p <0.05$). At 96 h, the percentage of cells had decreased to 25% regarding the initial amount under all treatments ($p <0.05$).

Due to our findings regarding cell proliferation, we decided to investigate whether individual or combined treatments induced cell death. Fig. 3A shows representative photomicrographs of the results corresponding to the identification of the type of cell death produced by the treatments. The first photomicrograph shows the staining of living cells in fluorescent green; cells in apoptosis, cells with defined morphology and nuclei show in a range from yellow to intense orange; and cells in necrosis, characterized by an undefined nucleus are fluorescent red. According to the images obtained, AmB-A21, like the other treatments, induced cell death via apoptosis. The morphology of cells was preserved even in the nucleus. Yellow to intense orange fluorescence, however, suggested early stages of apoptosis. This same situation was observed with AmB. However, cells treated with carboplatin had a higher number of cells in apoptosis and even necrosis, showing increased orange and red fluorescence, similar morphology had cells treated with AmB and AmB-A21 combined with carboplatin.

Fig. 3B also shows the quantification of cells in apoptosis under the different treatments. AmB-A21 alone induced death by apoptosis in 45% of the cell population ($p<0.05$). Cells treated with AmB and carboplatin alone showed a 63% and 70% increase in apoptosis, respectively ($p<0.05$). AmB-A21 produced 20% less apoptosis than AmB in A525 cells ($p<0.05$). However, when AmB-A21 was used along carboplatin showed a 39% increase ($p<0.05$), as compared with AmB-A21 alone. When AmB was combined with carboplatin increased the number of cells in apoptosis but did so by a mere 25% ($p<0.05$) when compared to cells treated with AmB alone.

Fig. 4A shows the results obtained from the DNA analysis in treated cells, either with single drugs or in combination. The first row shows the photomicrographs corresponding to DNA damage, which is indicated by red fluorescence. The second row shows the photomicrographs that correspond to the nucleus of the cells (blue fluorescence). The third row shows the merger of the 2 channels to demonstrate that red fluorescence corresponds to a cell and is not a source of noise. In a preliminary way, we can see that combined treatments led to greater DNA damage than individual treatments. AmB-A21 treated cells showed the least amount of damage. However, when cells were treated with AmB-A21 in combination with carboplatin, there was substantial DNA damage.

Fig. 4B shows the quantification of the corresponding DNA damage fluorescence. AmB-A21 in individual treatment produced minimal DNA damage (7%), as compared to the positive control ($p<0.05$). However, the combination of AmB-A21 with carboplatin did so 13.6 times compared to individual treatment ($p<0.05$). The combination of AmB-A21 with carboplatin potentiated DNA damage. On the other hand, when AmB was combined with carboplatin, a similar effect was observed ($p <0.05$).

Cells	CI	AmB	AmB-A21	Carboplatin
A549	CI_{25}	29 µg/mL	67 µg/mL	515 µg/mL
	CI_{50}	60 µg/mL	121 µg/mL	1031 µg/mL

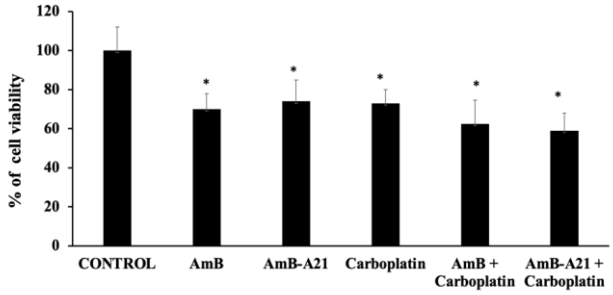


Fig. 1: Effect of AmB and AmB-A21 on the viability of A549 cells. * p<0.05 as compared with control; # p<0.05 as compared with AmB; $ p<0.05 as compared with AmB-A21; % p<0.05 as compared with carboplatin; & p<0.05 as compared with Carboplatin + AmB; @ as compared with carboplatin + AmB-A21.

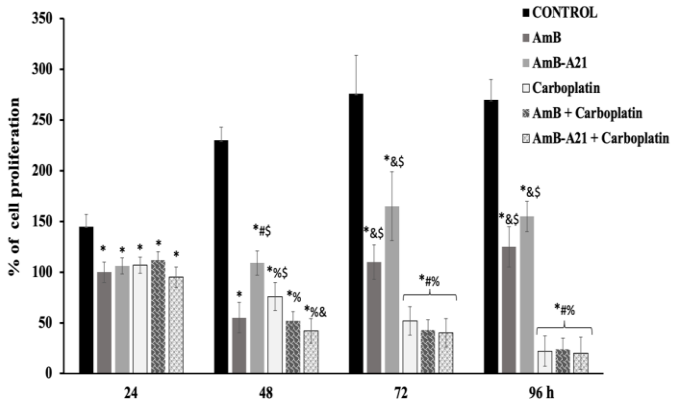
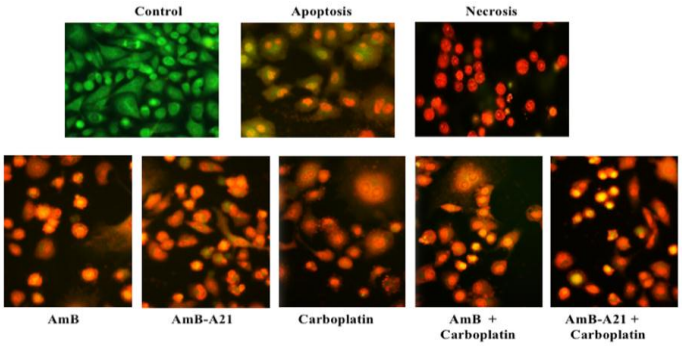


Fig. 2: Effect of AmB and AmB-A21 on the proliferation of A549 cells. * p<0.05 as compared with control; # p<0.05 as compared with AmB; % p<0.05 as compared with AmB-A21; & p<0.05 as compared with carboplatin; $ p<0.05 as compared with carboplatin + AmB.



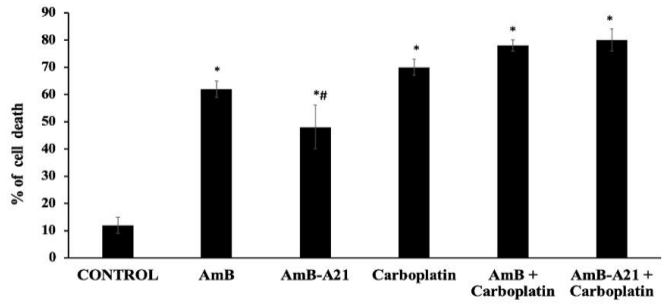


Fig. 3: Cell death induced by AmB and AmB-A21 in A549 cells. Representative microphotographs of cells treated with drugs alone or with carboplatin (40X). * p<0.05 as compared with control, # p<0.05 as compared with AmB, $ p<0.05 as compared with AmB-A21, & p<0.05 as compared with carboplatin, % p<0.05 as compared with AmB, @ as compared with carboplatin with AmB-A21.

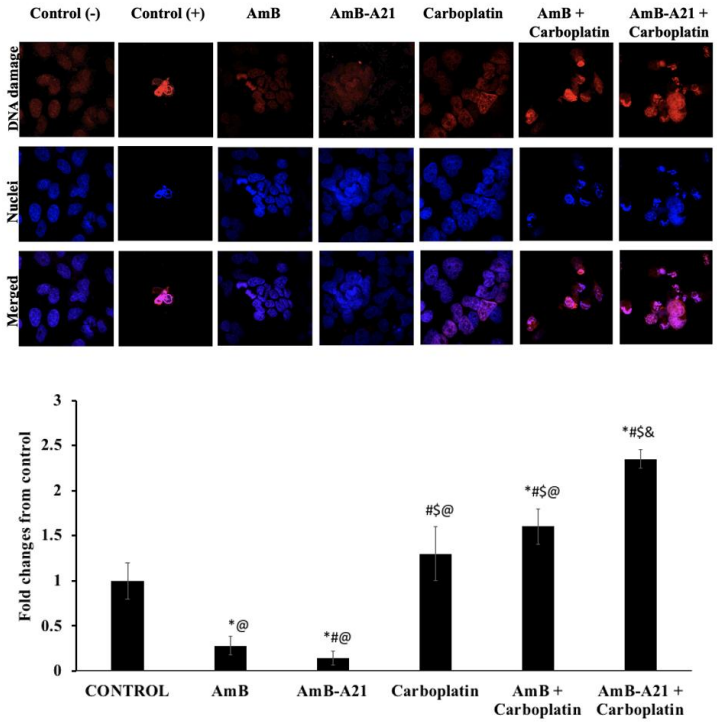


Fig. 4: DNA damage induced by AmB-A21 in A549 cells. Representative microphotography of A549 cells treated alone or in combination with carboplatin for 24 hours (60X) The lower * p<0.05 as compared with control; # p<0.05 as compared with AmB; $ p<0.05 as compared with AmB-A2; & p<0.05 as compared with carboplatin; % p<0.05 as compared with carboplatin with AmB; @ p<0.05 as compared with carboplatin with AmB-A21.

DISCUSSION

Lung cancer is a worldwide public health problem, and existing treatments are not sufficiently effective against the disease. For this reason, there is interest in evaluating new or existing drugs that can provide effective and safe therapeutic alternatives.

Our group sought to evaluate the antineoplastic capacity of AmB-A21 taking into consideration that the parent molecule (AmB) has been shown to have antineoplastic effects and a synergistic effect with conventional anticancer drugs.[11,14] Given AmB-A21 excellent safety margin, researching its potential antineoplastic effects would be very useful for the improvement of current antineoplastic treatments.

Our study showed AmB-A21 decreased cell viability by itself, and this effect increased when is used in combination with carboplatin. If we consider that IC_{25} were used, this indicates there was a synergistic effect between the drugs. Previous studies of AmB in combination with cisplatin, carboplatin, or doxorubicin have shown an increase in cytotoxicity of 25–30%.[24]

Although the concentration of AmB-A21 was 2 times higher than that of AmB, it also proved more effective at inducing apoptosis and inhibiting invasiveness. Tavanger et al., demonstrated that AmB was more effective as a pretreatment than when administered together with the antineoplastic.[14] Therefore, if AmB-A21 were used as a pretreatment, it could also exert a better pharmacological effect. One possible reason for the synergic effects of AmB-A21 and carboplatin is that perhaps there is no competitiveness among the mechanisms of action: AmB-A21 favors the formation of channels or pores in the fungus wall. It has been recently reported that AmB interacts with the lipid/sterol monolayer.[25] Therefore, if this mechanism occurs in the tumor cell, it could favor the entry of the antineoplastic, and thus enhance the effect of the antitumor therapy. AmB-A21, on the other hand, has a greater affinity to membranes containing ergosterol rather than cholesterol and therefore has reduced effects.[21] which indicates it must have other mechanisms of interaction with mammalian cells.

One of the characteristics considered indicative of malignancy is the proliferative capacity of the tumor cell. Therefore, we thought it important to assess whether AmB-A21 could reduce cell proliferation and whether carboplatin enhanced these effects. As observed in the proliferation results, the only drug that effectively inhibited proliferation was AmB-A21. The other

treatments inhibited proliferation during the first 24 h, but subsequently likely caused cell death given they were below the starting point. Previous studies report AmB can reduce the proliferation of Raji cells by 20%.[12] However, its effects were only partial in the present study. One of the possible reasons for the decreased proliferation is antineoplastic drugs'[26] and AmB's[10] proven ability to induce cell death. Cytotoxic effects were best demonstrated when cells were exposed to combined treatments.

Our analysis of cell death showed all drugs induced apoptosis, although to a different degree. AmB-A21 acted synergically with carboplatin. The same situation was observed with AmB. It is well known that chemotherapeutic anti-cancer drugs induce cell death via apoptosis, which would perforce decrease the overall number of cells, as observed in the proliferation assay. It has also been reported that AmB produces pores in the cell membrane and induces the opening of potassium channels.[27] Therefore, the mechanism of action of antifungal agents could be favoring intracellular ionic alteration, modifying the pro- and anti-apoptotic signaling pathways.[28] However, that effect has not yet been attributed to either AmB or AmB-A21 in mammalian cells.

This study evaluated treatment-caused DNA damage (by double strand break). AmB-A21 caused the least DNA damage. However, when AmB-A21 was combined with carboplatin, DNA damage was more evident than when cells were treated with antineoplastic alone. Carboplatin is a potent alkylating drugs that stimulate genomic instability and cell death by apoptosis.[29,30] AmB has also been reported to cause DNA damage by double-strand breaks and fragmentation of DNA.[31,32] We propose that AmB-A21 might have the same effect. However, the reason for AmB-A21's potentiated combination with antineoplastic drugs still needs to be elucidated.

CONCLUSION

Based on our results, it can be concluded that AmB-A21 has effective antitumor properties. The combination of AmB-A21 with carboplatin increases the effect of the single drug treatments. Therefore, AmB-A21 could be considered a therapeutic option for lung cancer treatment. The use of AmB-A21 with other antineoplastic drugs could reduce doses and side effects, improving patients' quality of life.

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