

Bumetanide annihilation of amphotericin B-induced apoptosis and cytotoxicity is due to its effect on cellular K⁺ flux

L. Marklund^a, P. Behnam-Motlagh^{a,b}, R. Henriksson^b and K. Grankvist^{a*}

Departments of ^aClinical Chemistry and ^bOncology, Umeå University, S-901 87 Umeå, Sweden

The antifungal antibiotic amphotericin B causes considerable toxic effects during clinical therapy. We have shown previously that amphotericin B-induced cytotoxicity and apoptosis were eradicated by the Na⁺, K⁺, 2Cl⁻ cotransport inhibitor bumetanide. To elucidate the role of K⁺ flux and the activity of Na⁺, K⁺ ATPase and Na⁺, K⁺, 2Cl⁻ cotransport in apoptosis and cytotoxicity induced by amphotericin B alone and combined with bumetanide, we quantified the influx and efflux of K⁺ of mesothelioma cells (P31) using the K⁺ analogue ⁸⁶Rb⁺ with ouabain (100 µmol/L) as the K⁺ influx probe. To determine the susceptibility of *Candida albicans* to amphotericin B when combined with bumetanide we used a plate diffusion method. Amphotericin B or bumetanide alone significantly stimulated ⁸⁶Rb⁺ efflux during the first 15 min. However, when added simultaneously, the cellular ⁸⁶Rb⁺ efflux was markedly decreased. Amphotericin B (3 mg/L) had no effect on immediate (15 min) total ⁸⁶Rb⁺ influx. When bumetanide (100 µmol/L) was added, the total ⁸⁶Rb⁺ influx was markedly reduced due to inhibition of augmented Na⁺, K⁺, 2Cl⁻ cotransport and low Na⁺, K⁺ ATPase activity. Bumetanide did not affect the susceptibility of *C. albicans* to amphotericin B, which suggests that bumetanide or related drugs could be used in antifungal therapy to increase amphotericin B effectiveness without increasing its adverse effects. We suggest that bumetanide hampering of amphotericin B-induced cytotoxicity and apoptosis could be due to an immediate reduction of cellular K⁺ efflux as well as disordered K⁺ influx.

Introduction

Amphotericin B is a polyene antifungal antibiotic frequently used in the chemotherapy of fungal infections. However, amphotericin B exhibits poor effectiveness and high toxicity. The need for new effective and non-toxic antimycotic therapy has deepened with the spread of HIV infection leading to a growing number of immunocompromised patients, and with the appearance of multidrug-resistant fungal strains.

Amphotericin B creates cell membrane pores^{1–3} inducing K⁺ efflux and apoptosis in eukaryotic cells.^{4,5} We have shown previously⁵ that amphotericin B-induced apoptosis and cytotoxicity were considerably reduced by the Na⁺, K⁺, 2Cl⁻ cotransport blocker bumetanide.

Efflux of K⁺ plays an important role during the apoptotic process. The transition of an apoptotic cell from a state of a high towards a low K⁺ content permits both the

loss in cell volume and the activation of enzymes that mediate induction of apoptosis.⁶ K⁺ uptake mechanisms, such as Na⁺, K⁺ ATPase or Na⁺, K⁺, 2Cl⁻ cotransport, are activated to compensate the loss of intracellular K⁺,⁷ and thereby counteract apoptosis.⁸ Modulation of potassium pumps could thus be expected to decrease or increase drug-induced apoptosis and cytotoxicity.

To clarify further the role of K⁺ flux in amphotericin B-induced apoptosis and cytotoxicity, we used the potassium analogue rubidium (⁸⁶Rb⁺) to measure direct effects on K⁺ efflux and influx. The Na⁺, K⁺ ATPase blocker ouabain was used as a probe for determination of Na⁺, K⁺ ATPase and Na⁺, K⁺, 2Cl⁻ cotransport activity on the studied human mesothelioma cell line (P31) known to express these two K⁺ uptake mechanisms.⁹ The question of whether addition of bumetanide influenced amphotericin B-induced cytotoxicity to *Candida albicans* fungal cells was examined with a plate diffusion method.

*Corresponding author. Tel/Fax: +46-90-785-12-62; E-mail: kjell.Grankvist@klinkemi.umu.se

Materials and methods

Cell culture

A human pulmonary mesothelioma cell line (P31) propagated under standard tissue culture conditions was used. The cells were grown as monolayer culture in Eagle's minimal essential medium (EMEM) in Earl's saline supplemented with 10% fetal calf serum, 200 $\mu\text{mol/L}$ L-glutamine, penicillin and streptomycin. They were incubated at 37°C in a humidified atmosphere containing 5% CO_2 .

$^{86}\text{Rb}^+$ efflux

The P31 cells were grown for 24 h in the wells of 24-well plates as monolayer culture in EMEM in Earl's saline supplemented with 10% fetal calf serum, 200 $\mu\text{mol/L}$ L-glutamine and 1 $\mu\text{mol/L}$ $^{86}\text{RbCl}$ (500 Ci/mol). They were incubated at 37°C in a humidified atmosphere containing 5% CO_2 . The medium was then discarded by aspiration and the plates washed once with EMEM without fetal calf serum. EMEM was then added to the wells, with or without test substances (final concentrations: 3 mg/L amphotericin B and/or 100 $\mu\text{mol/L}$ bumetanide), and the incubation continued for 15 or 60 min. The supernatant of each well was then aspirated and added to labelled scintillation vials. The remaining adherent cells were trypsinized, the well content transferred to scintillation vials, and radioactivity determined by liquid scintillation counting. All wells were handled in parallel in each experiment and with the same number of cells/well.

$^{86}\text{Rb}^+$ influx and methodology for interpretation of results with channel-blocking agents

The pulmonary carcinoma cells were grown in 24-well cluster plates for 0 or 60 min in EMEM (containing 5.3 mmol/L K^+), without (control) or with amphotericin B (3 mg/L) alone or combined with bumetanide (100 $\mu\text{mol/L}$), and then incubated for 15 min in the same medium supplemented with 1 $\mu\text{mol/L}$ $^{86}\text{RbCl}$ (500 Ci/mol), with or without ouabain (100 $\mu\text{mol/L}$), alone or in combination. The concentrations of the channel-blocking agents were chosen because of the total inhibition of Na^+ , K^+ , 2Cl^- cotransport and Na^+ , K^+ ATPase activity.⁹ The cells were rinsed, trypsinized and transferred to scintillation vials, and radioactivity was determined by liquid scintillation counting. Isotope influx in drug-treated cells was expressed as a percentage of the influx of controls (basal influx) handled in parallel and without test substances. Counting ensured that the number of cells treated with drugs at 60 min were not reduced in number compared with untreated control cells. From a previous study⁹ we learned that three types of potassium flux pathway exist in this pulmonary carcinoma cell line: Na^+ , K^+ , 2Cl^- cotransport (blocked by bumetanide), Na^+ , K^+ ATPase activity (blocked by ouabain) and high conductance K^+ channels (blocked by tetraethyl-

ammonium, TEA). As the TEA effect on $^{86}\text{Rb}^+$ influx of P31 cells was small (and in the present experiments almost negligible), with the remnant $^{86}\text{Rb}^+$ influx c. 3% of control when combining test substances with ouabain and bumetanide simultaneously, we interpreted the reduction of $^{86}\text{Rb}^+$ influx (in relation to untreated control) when adding ouabain (ouabain-sensitive $^{86}\text{Rb}^+$ influx) as being due to Na^+ , K^+ ATPase activity, and the $^{86}\text{Rb}^+$ influx remaining (ouabain-insensitive $^{86}\text{Rb}^+$ influx) minus the remnant $^{86}\text{Rb}^+$ influx in the presence of both ouabain and bumetanide as being due solely to Na^+ , K^+ , 2Cl^- cotransport activity.

Plate diffusion method

To measure fungal susceptibility to amphotericin B alone and in combination with bumetanide we used a plate diffusion method. Two colonies of *C. albicans* were suspended in 5 mL of Milli-Q and transferred to a buffered YNB plate. Amphotericin B (100 μL , final concentration 3 mg/L; $n = 6$), alone or in combination with rising concentrations of bumetanide (1, 10, 100 $\mu\text{mol/L}$; $n = 2$), standard solution and internal standard solution were added to 10 mm holes punched in the plate. The plates were incubated for 30 min at room temperature, then further incubated at 37°C overnight. The size of the inhibition zones produced by the different solutions was measured and compared with amphotericin B alone.

Chemicals

Amphotericin B (CAS 1397-89-3) lot no. 70316 was from Bristol-Myers Squibb, New York, NY, USA. Ouabain (CAS 11018-89-6), bumetanide (CAS 28395-03-1) and tetraethylammonium chloride (CAS 56-34-8) were from Sigma Chemical Company, St Louis, MO, USA. $^{86}\text{RbCl}$ (CAS 7791-11-9) was purchased from Amersham International, Amersham, UK. EMEM and L-glutamine were from Gibco Ltd, Paisley, UK. Fetal calf serum was purchased from Biochrom KG, Berlin, Germany. Cell culture 24-well cluster plates and polystyrene dishes were from Costar, Cambridge, MA, USA. All other chemicals were of analytical grade.

Statistics

Statistical significance of the differences between surviving clones was tested with one-way ANOVA. The level of significance for rejecting the null hypothesis of zero treatment effect was $P = 0.05$.

Results

Effects of amphotericin B and bumetanide on $^{86}\text{Rb}^+$ efflux

The $^{86}\text{Rb}^+$ efflux in P31 pulmonary mesothelioma cells was run for 15 or 60 min. Therefore, we assumed that the efflux

Amphotericin B toxicity and cellular potassium flux

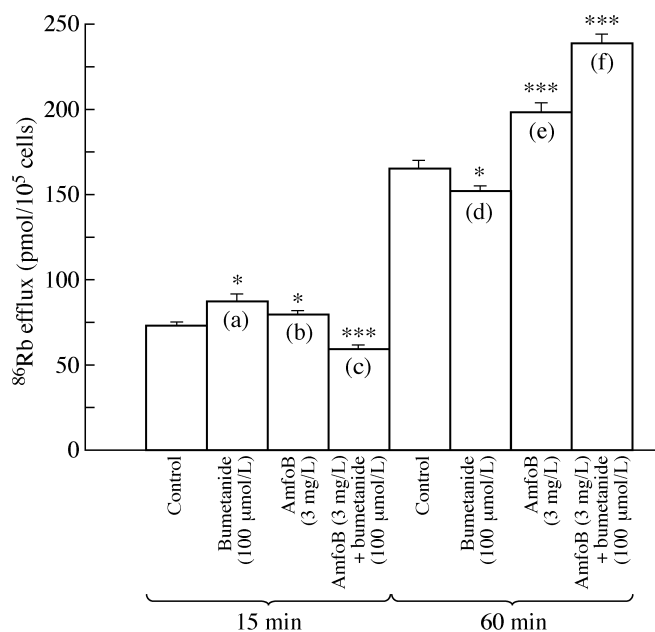


Figure. ⁸⁶Rb efflux of cells first grown for 24 h with 1 μmol/L ⁸⁶RbCl, rinsed with culture medium and then incubated for 15 or 60 min with or without test substances (final concentrations: 3 mg/L amphotericin B, 100 μmol/L bumetanide) alone or in combination. The supernatant was then aspirated and radioactivity determined by liquid scintillation counting. ⁸⁶Rb isotope efflux of drug-treated cells was expressed as a percentage of total isotope loaded. Data denote mean ± S.E.M. for 12–20 separate observations. **P* < 0.05; ****P* < 0.001. (a and b) Compared with 15 min control; (c) compared with 15 min with amphotericin B alone; (d and e) compared with 60 min control; (f) compared with 60 min with amphotericin B alone.

during the first 15 min reflects the efflux rate of ⁸⁶Rb⁺ and is not affected by re-uptake of extracellular accumulation of the isotope. ⁸⁶Rb⁺ efflux during 60 min, however, could possibly be counteracted by ⁸⁶Rb⁺ influx due to extracellular accumulation.

Amphotericin B (3 mg/L) or bumetanide (100 μmol/L) significantly (*P* < 0.05) stimulated the immediate (15 min) ⁸⁶Rb⁺ efflux from 72.2 ± 2.4 (control) to 78.8 ± 1.9 and 86.1 ± 5.3 pmol/10⁵ cells/15 min, respectively (Figure). However, when added together, amphotericin B/bumetanide initially reduced cellular ⁸⁶Rb⁺ efflux markedly and significantly (*P* < 0.001) to 57.8 ± 2.4 pmol/10⁵ cells/15 min, which is even below the level of untreated control cells. When the ⁸⁶Rb⁺ efflux was run for 60 min, bumetanide reduced (*P* < 0.05) efflux [from 164.8 ± 4.6 (control) to 151.5 ± 3.0 pmol/10⁵ cells/60 min]. The augmented efflux induced by amphotericin B was even more pronounced (*P* < 0.001) after 60 min efflux, but now the amphotericin B/bumetanide mixture augmented (from 197.8 ± 5.6 to 238.3 ± 3.9 pmol/10⁵ cells/60 min; *P* < 0.001) the efflux seen with amphotericin B alone (Figure).

Effect of amphotericin B on immediate ⁸⁶Rb⁺ influx

The ⁸⁶Rb⁺ influx in P31 lung mesothelioma cells was linear for 15 min. Therefore, it was assumed that the influx during this time period reflects the influx rate of ⁸⁶Rb⁺ and is not affected by intracellular accumulation of the isotope.⁹ Addition of TEA (a blocker of K⁺ channels with high conductance) to the ouabain and bumetanide combination only marginally affected the ⁸⁶Rb⁺ influx (results not shown). The absolute influx of ⁸⁶Rb⁺ of control cells was 74.4 ± 0.8 pmol/10⁵ cells/15 min. Ouabain (100 μmol/L) inhibited the influx to 23.0 ± 3.6 pmol/10⁵ cells/15 min, thus yielding an Na⁺, K⁺ ATPase activity of 51.4 (74.4–23.0) pmol/10⁵ cells/15 min and an Na⁺, K⁺, 2Cl⁻ cotransport activity (ouabain-insensitive ⁸⁶Rb⁺ influx) of 20.4 (23.0–2.6) pmol/10⁵ cells/15 min, when remnant influx (2.6 pmol/10⁵ cells/15 min) of the combination of ouabain and bumetanide was deducted. This remnant ⁸⁶Rb⁺ influx was not affected (influx 2.6–3.5 pmol/10⁵ cells/15 min) by the addition of amphotericin B. This indicates that Na⁺, K⁺, 2Cl⁻ cotransport and Na⁺, K⁺ ATPase activity are responsible for almost all ⁸⁶Rb⁺ influx in this cell line and that K⁺ channels with high conductance (TEA-blockable) were not activated during experiments. Bumetanide (100 μmol/L) inhibited ⁸⁶Rb⁺ influx to 51.8 ± 3.6 pmol/10⁵ cells/15 min, indicating a (bumetanide-sensitive) Na⁺, K⁺, 2Cl⁻ cotransport activity of 22.6 (74.4–51.8) and an Na⁺, K⁺ ATPase activity of 49.2 (51.8–2.6) pmol/10⁵ cells/15 min (Table 1). The similar results obtained on cotransport and ATPase activity, whether bumetanide or ouabain was used as the probe for the activity determination, shows that the probes are specific inhibitors of cotransport and ATPase activity in the control situation without amphotericin B.

Amphotericin B (3 mg/L) only slightly (76.5 ± 0.8 pmol/10⁵ cells/15 min) increased the immediate (15 min) ⁸⁶Rb⁺ influx (Table 1), and when combined with ouabain seemed to be due to a slight increase in Na⁺, K⁺ ATPase activity (56.8 pmol/10⁵ cells/15 min) and slight decrease (16.2 pmol/10⁵ cells/15 min) in Na⁺, K⁺, 2Cl⁻ cotransport activity. Amphotericin B combined with bumetanide markedly reduced ⁸⁶Rb⁺ influx to 42.8 ± 1.5 pmol/10⁵ cells/15 min, indicating an Na⁺, K⁺, 2Cl⁻ cotransport activity of 33.7 (76.5–42.8) pmol/10⁵ cells/15 min and an Na⁺, K⁺ ATPase activity of 39.3 pmol/10⁵ cells/15 min (Table 1). However, when adding ouabain to amphotericin B, ⁸⁶Rb⁺ influx decreased to 19.7 ± 1.5, indicating an Na⁺, K⁺ ATPase activity of 56.8 pmol/10⁵ cells/15 min and an Na⁺, K⁺, 2Cl⁻ cotransport activity of 16.2 pmol/10⁵ cells/15 min.

⁸⁶Rb⁺ influx after 60 min incubation with amphotericin B

Addition of TEA (a blocker of K⁺ channels with high conductance) to the ouabain and bumetanide combination only marginally reduced the ⁸⁶Rb⁺ influx (from 4.0 ± 0.07

to 3.5 ± 0.20 pmol/ 10^5 cells/15 min; $n = 6$) after 60 min incubation.

$^{86}\text{Rb}^+$ influx of control cells with or without ouabain and/or bumetanide was very similar to the results of the shorter 15 min incubation (Table 2). However, after incubation with amphotericin B for 60 min, $^{86}\text{Rb}^+$ influx activity was increased to 88.8 ± 1.9 pmol/ 10^5 cells/15 min. Using ouabain as a probe showed that this was due to a huge (from 51.3 to 78.0 pmol/ 10^5 cells/15 min) activation of Na^+ , K^+ ATPase, but a reduced (7.4 pmol/ 10^5 cells/15 min) Na^+ , K^+ , 2Cl^- cotransport activity. The combination of amphotericin B with bumetanide incubated for 60 min showed similar effects on Na^+ , K^+ ATPase and Na^+ , K^+ , 2Cl^- cotransport activity results to amphotericin combined with ouabain (Table 2).

Fungal susceptibility to amphotericin B alone and in combination with bumetanide

The mean value ($n = 6$) of the inhibition zones by amphotericin B (3 mg/L) was 23 mm. When bumetanide was added in rising concentrations (1, 10, 100 $\mu\text{mol/L}$), the inhibition zones were 23.7, 22.7 and 22.7 mm, respectively (mean value, $n = 2$), thus there was no significant difference compared with amphotericin B alone.

Discussion

We have shown previously that amphotericin B-induced cytotoxicity and apoptosis are eradicated by the Na^+ , K^+ , 2Cl^- cotransport inhibitor bumetanide.⁵ In the present study, we showed that amphotericin B significantly increased the efflux of the K^+ analogue $^{86}\text{Rb}^+$ on cultured P31 pulmonary mesothelioma cells. When bumetanide was added to the amphotericin B-treated cells the 15 min efflux of $^{86}\text{Rb}^+$ was markedly reduced. The initial effect of amphotericin B combined with bumetanide on $^{86}\text{Rb}^+$ influx demonstrated a stimulation of Na^+ , K^+ , 2Cl^- cotransport activity, whereas Na^+ , K^+ ATPase activity was lowered. This indicates that the inhibitory effect of bumetanide on amphotericin B cytotoxicity and apoptosis could be due to an initial inhibition of K^+ efflux of the drugs combined with disordered K^+ influx. Since the inhibition zones in the plate diffusion test with *C. albicans* showed no significant difference when bumetanide was added compared with amphotericin B alone, we conclude that the susceptibility of the fungus to amphotericin B is not affected by bumetanide.

Despite its considerable toxic effects, amphotericin B is the drug of choice in the treatment of systemic fungal infections. During therapy there are difficulties in obtaining adequate fungistatic plasma levels of amphotericin B without causing undesired side effects such as nephrotoxicity,¹⁰ or electrolyte abnormalities. Clinically, amphotericin B levels of *c.* 4.3 $\mu\text{mol/L}$ (4 mg/L) have been obtained.¹¹ Amphotericin B produces pores in the membrane,¹⁻³

inducing potassium efflux, and increases by a factor of six the opening probability of potassium channels.¹² A non-toxic concentration of amphotericin B significantly increased the endothelial cell membrane permeability to K^+ .¹³ Leakage of K^+ within a few minutes was also found in rat hepatocytes.¹⁴

Transmembrane fluxes of cations are involved in several important cell processes, including cell proliferation, volume regulation and apoptosis.⁸ Efflux of K^+ is fundamental during the apoptotic process since potassium loss causes the volume reduction of the cell during apoptosis. Low intracellular potassium concentration seems to be important for the activation of enzymes mediating apoptosis, and similarly physiological levels of K^+ inhibit enzyme activation.^{6,15} Potassium efflux provoked by using NMDA-receptor gated K^+ efflux, K^+ ionophores or K^+ channel openers promotes neural apoptosis.^{16,17} Thus, modulation of transmembrane K^+ flux and thereby the intracellular potassium content would influence the induction of apoptosis and cytotoxicity of various drugs, including amphotericin B.

The major ion transport system accomplishing K^+ uptake in shrunken cells and thereby counteracting apoptosis is the Na^+ , K^+ , 2Cl^- cotransporter. K^+ accumulation is inhibited by blockers of cotransport, thereby leading to increased intracellular K^+ loss.¹⁸⁻²⁰ Surprisingly we found that the Na^+ , K^+ , 2Cl^- cotransport inhibitor bumetanide markedly reduced the cytotoxicity of amphotericin B from 64% to 86% surviving clones of P31 cells. The combination of amphotericin B with bumetanide almost annihilated the apoptotic index ($P < 0.05$) in the quantitative nucleosome ELISA assay.⁵ Unexpectedly, however, the combination of bumetanide and amphotericin B initially reduced $^{86}\text{Rb}^+$ efflux even below the $^{86}\text{Rb}^+$ efflux of control cells. Thus, the elimination of amphotericin B cytotoxicity and apoptosis by bumetanide may be due to inhibition of potassium efflux rather than to inhibition of Na^+ , K^+ , 2Cl^- cotransport activity. Whether or not the annihilating interaction of bumetanide with amphotericin B cytotoxicity and apoptosis is specific for bumetanide and related to Na^+ , K^+ , 2Cl^- cotransport activity has to be further elucidated by investigating the dose-response relationship of bumetanide and analogues on cytotoxicity, apoptosis, cell size and $^{86}\text{Rb}^+$ flux. The combinatory effect of bumetanide and amphotericin B could be interpreted as initial blocking of stimulated Na^+ , K^+ , 2Cl^- cotransport activity with a simultaneous reduction of Na^+ , K^+ ATPase activity. However, as bumetanide in some situations is also known to inhibit Na^+ , K^+ ATPase activity, an alternative interpretation of stimulated Na^+ , K^+ , 2Cl^- cotransport activity would be that the combination of bumetanide with amphotericin B in addition to blockage of Na^+ , K^+ , 2Cl^- cotransport activity also partly inhibits Na^+ , K^+ ATPase activity. The alternatives could perhaps be resolved only by recording cell volume measurements concomitantly with $^{86}\text{Rb}^+$ flux determinations.

Amphotericin B toxicity and cellular potassium flux

Table 1. $^{86}\text{Rb}^+$ influx (pmol/ 10^5 /cells/15 min) of lung mesothelioma cell line P31 cells in medium only (control), or medium with 3 mg/L amphotericin B, bumetanide (100 $\mu\text{mol/L}$) and/or ouabain (100 $\mu\text{mol/L}$) alone or in combination for 15 min ($n = 10$)

Combination of drugs	^{86}Rb influx		
	total	ouabain-insensitive (bumetanide-sensitive)	ouabain-sensitive (bumetanide-insensitive)
Control	74.4 \pm 0.8		
Ouabain	23.0 \pm 3.6	20.4 (22.6)	51.4 (49.2)
Bumetanide	51.8 \pm 3.6		
Ouabain + bumetanide	2.6 \pm 0.1		
AmB	76.5 \pm 0.8		
AmB + ouabain	19.7 \pm 1.2	16.2 (33.7)	56.8 (39.3)
AmB + bumetanide	42.8 \pm 1.5		
AmB + bumetanide + ouabain	3.5 \pm 0.1		

AmB, amphotericin B.

Table 2. $^{86}\text{Rb}^+$ influx (pmol/ 10^5 /cells/15 min) of lung mesothelioma cell line P31 cells in medium only (control), or medium with 3 mg/L amphotericin B and/or bumetanide (100 $\mu\text{mol/L}$) for 75 min

Combination of drugs	^{86}Rb influx		
	total	ouabain-insensitive (bumetanide-sensitive)	ouabain-sensitive (bumetanide-insensitive)
Control	74.4 \pm 0.8		
Ouabain	23.1 \pm 1.6	20.0 (22.5)	51.3 (48.8)
Bumetanide	51.9 \pm 1.0		
Ouabain + bumetanide	3.1 \pm 0.1		
AmB	88.8 \pm 1.9		
AmB + ouabain	10.8 \pm 0.6	7.4 (10.7)	78.0 (74.7)
AmB + bumetanide	78.1 \pm 2.3		
AmB + ouabain + bumetanide	3.4 \pm 0.1		

AmB, amphotericin B.
 $^{86}\text{Rb}^+$ was added with or without ouabain (100 $\mu\text{mol/L}$) during the last 15 min of the incubation and $^{86}\text{Rb}^+$ influx during the last 15 min of the incubation calculated ($n = 10$).

The preserved antifungal effect implies a clinical application of the Na⁺, K⁺, 2Cl⁻ cotransport blocker bumetanide, or related drugs in antifungal therapy, to increase amphotericin B effectiveness without increasing its adverse effects. The induction of apoptosis seems to be of significant importance in amphotericin B-induced cytotoxicity. We have shown previously that the Na⁺, K⁺, 2Cl⁻ cotransport inhibitor bumetanide eradicated amphotericin B-induced cytotoxicity and apoptosis.⁹ When Na⁺, K⁺, 2Cl⁻ cotransport activity of amphotericin B-treated cells is blocked by bumetanide, K⁺ efflux is considerably reduced. Thus, bumetanide reduction of amphotericin B cytotoxicity and apoptosis requires preserved intracellular K⁺ levels, and may be due to an initial reduction of cellular K⁺ efflux as well as a derangement of cellular K⁺ influx.

Acknowledgements

This investigation was supported by grants from the Swedish Cancer Society and the Lion's Cancer Research Foundation, Umeå University, Umeå, Sweden.

References

1. Fujii, G., Chang, J.-E., Coley, T. & Steere, B. (1997). The formation of amphotericin B channels in lipid bilayers. *Biochemistry* **36**, 4959–68.
2. Hsu, S.-F. & Burnette, R. (1997). Characterisation of the effects of amphotericin B on ion channels in MDCK cells using the patch-clamp technique. *Biochimica et Biophysica Acta* **1329**, 26–38.
3. Ramos, H., Valdivieso, E., Gamargo, F., Dagger, B. E. & Cohen, B. E. (1996). Amphotericin B kills unicellular leishmanias by forming aqueous pores permeable to small cations and anions. *Journal of Membrane Biology* **152**, 65–7.
4. Reinach, P. S. & Schoen, H. F. (1990). NPPB inhibits the basolateral membrane K⁺ conductance in the isolated bullfrog cornea. *Biochimica et Biophysica Acta* **1026**, 13–20.
5. Marklund, L., Henriksson, R. & Grankvist, K. (2000). Amphotericin B-induced apoptosis and cytotoxicity is prevented by the Na⁺, K⁺, 2Cl⁻ cotransport blocker bumetanide. *Life Science* **66**, 319–24.
6. Bortner, C. D., Hughes, F. M., Jr & Cidlowski, J. A. (1997). A primary role for K⁺ and Na⁺ efflux in the activation of apoptosis. *Journal of Biological Chemistry* **272**, 32436–42.
7. Hoffman, E. K. & Dunham, P. B. (1995). Membrane mechanisms and intracellular signalling in cell volume regulation. *International Review of Cytology—A Survey of Cell Biology* **161**, 173–242.
8. Lang, F., Busch, G. L., Ritter, M., Volkl, H., Waldegger, S., Gulbins, E. *et al.* (1998). Functional significance of cell volume regulatory mechanisms. *Physiological Reviews* **78**, 247–306.
9. Sandström, P.-E., Jonsson, Ö., Grankvist, K. & Henriksson, R. (1994). Identification of potassium flux pathways and their role in the cytotoxicity of estramustine in human glioma, prostatic and pulmonary carcinoma cell lines. *European Journal of Cancer* **12**, 1822–6.
10. Sabra, R. & Bransch, R. A. (1990). Amphotericin B nephrotoxicity. *Drug Safety* **5**, 94–108.
11. Atkinson, A. J. & Bennet, J. E. (1978). Amphotericin B pharmacokinetics in human. *Antimicrobial Agents and Chemotherapy* **13**, 271–6.
12. Hsu, S. F. & Burnette, R. R. (1997). The effect of amphotericin B on the K-channel activity of MDK cells. *Biochimica et Biophysica Acta* **1152**, 189–91.
13. Cutaia, M., Bullard, S. R., Rudio, K. & Rounds, S. (1993). Characteristics of amphotericin B-induced endothelial cell injury. *Journal of Laboratory and Clinical Medicine* **121**, 244–56.
14. Binet, A. & Bolard, J. (1988). Recovery of hepatocytes from attack by the pore former amphotericin B. *Biochemical Journal* **253**, 435–40.
15. Montauge, J. W., Bortner, C. D., Hughes, F. M., Jr & Cidlowski, J. A. (1999). A necessary role for reduced intracellular potassium during the DNA degradation phase of apoptosis. *Steroids* **64**, 563–9.
16. Yu, S. P., Yeh, C. H., Sensi, S. L., Gwag, B. J., Canzoniero, L. M., Farhangrazi, Z. S. *et al.* (1997). Mediation of neuronal apoptosis by enhancement of outward potassium current. *Science* **278**, 114–7.
17. Lang, F., Ritter, M., Woll, E., Yu, S. P., Yeh, C., Strasser, U. *et al.* (1999). NMDA receptor-mediated K⁺ efflux and neuronal apoptosis. *Science* **284**, 336–9.
18. Barbiero, G., Duranti, F., Bonelli, G., Amenta, J. S. & Baccino, F. M. (1995). Intracellular ionic variations in the apoptotic death of L cells by inhibitors of cell cycle progression. *Experimental Cell Research* **217**, 410–8.
19. Lytle, C. (1997). Activation of the avian erythrocyte Na-K-Cl cotransport protein by cell shrinkage, cAMP, fluoride, and calyculin-A involves phosphorylation at common sites. *Journal of Biological Chemistry* **272**, 15069–77.
20. Greger, R., Heitzmann, D., Hug, M. J., Hoffmann, E. K. & Bleich, M. (1999). The Na⁺2Cl⁻K⁺ cotransporter in the rectal gland of *Squalus acanthias* is activated by cell shrinkage. *European Journal of Physiology* **438**, 165–76.

Received 11 April 2001; returned 5 July 2001; revised 21 August 2001; accepted 30 August 2001