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Pharmacological Modulation of Lung Cancer Cells for Potassium Ion Depletion

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Abstract. *Background: Depletion of intracellular potassium ions (K^+) is necessary for cells to shrink, induce DNA fragmentation and activate caspases, events which are features of apoptosis. Materials and Methods: We used $^{86}Rb^+$ as a K^+ analogue to evaluate the possibility of pharmacologically depleting human pulmonary mesothelioma (P31) and small cell lung cancer (U1690) cells of K^+, for future use in studies of apoptosis induction. Results: The Na^+, K^+, $2Cl^-$-cotransport inhibitor bumetanide transiently inhibited $^{86}Rb^+$ influx, but when combined with the Na^+, K^+, ATPase pump inhibitor ouabain there was a marked and lasting (up to 6 h) $^{86}Rb^+$ influx inhibition. Cellular K^+ efflux was augmented by amphotericin B, digitonin and nigericin. Amphotericin B was an effective $^{86}Rb^+$ efflux stimulator with low cytotoxicity, whereas digitonin caused cell detachment and nigericin increased LDH release in the U1690 cell line, indicating considerable toxicity of the drugs. Conclusion: It is possible to efficiently reduce intracellular K^+ by persistent K^+ influx inhibition and simultaneous K^+ efflux stimulation with clinically available drugs.*

Many anticancer drugs exert their effect by stimulating the rate of apoptosis. Apoptosis is accompanied by characteristic morphological changes, such as cell shrinkage, membrane blebbing, DNA fragmentation and formation of apoptotic bodies. The transition of an apoptotic cell from a state of high towards a low potassium ion (K^+) content is an early and necessary event for cell volume reduction and activation of enzymes that mediate the induction of apoptosis (1). The events triggering intracellular K^+ depletion during apoptosis are unknown. Provoked K^+ efflux by activation of K^+ channels or by K^+ ionophores promotes apoptosis (2-4), whereas blockage of K^+ efflux channels attenuates apoptosis (4, 5). K^+ uptake mechanisms, such as Na^+, K^+, $2Cl^-$-cotransport and Na^+, K^+, ATPase pump activity, are activated in order to compensate for the loss of intracellular K^+ (6-8) and, thereby, counteract apoptosis (9). Stimulation of K^+ efflux and/or inhibition of K^+ reuptake mechanisms could therefore be expected to increase the drug-induced apoptosis of chemotherapeutic agents and, subsequently, enhance their tumoricidal effects.

Only a few articles have described K^+ flux mechanisms and the pharmacological manipulation of K^+ fluxes of cancer cells. Our studies on the mesothelioma cell line P31 have shown that Na^+, K^+, ATPase and Na^+, K^+, $2Cl^-$-cotransport are responsible for almost all of the influx of potassium ions (10). We recently showed that the antifungal ionophore amphotericin B, together with the Na^+, K^+, $2Cl^-$-cotransport inhibitor bumetanide, increased efflux and reduced K^+ influx, leading to enhanced cisplatin-induced apoptosis in P31 cells, whereas the effect of other K^+ influx inhibitors or K^+ efflux stimulators are unknown (11).

The $^{86}Rb^+$ method is an exquisite K^+ channel function assay that gives more information about the K^+ fluxes in a population of cells than the whole cell voltage clamp technique that is often used in characterization studies (12). To our knowledge, no systematic studies of pharmacological agents which affect tumour cell K^+ influx and efflux in order to deplete the cells of K^+ have been performed. We therefore used the $^{86}Rb^+$ method to determine how to pharmacologically stimulate K^+ efflux and inhibit K^+ influx in mesothelioma and small cell lung cancer cells. The results are the base for future attempts to enhance apoptosis during cancer treatment.

Materials and Methods

A human pulmonary mesothelioma cell line (P31) (13) and a small cell lung cancer cell line (U1690) (14) were used. The cells were

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Table I. *Ionophores tested for K^+ efflux stimulation.*

Ionophore	Concentrations used	References
Amphotericin B	3, 6 and 9 mg/L	(36)
Beauvericin	5, 10 and 25 μmol/L	(24, 28)
Digitonin	7.5, 15 and 30 mg/L	(25)
Gramicidin D	0.01, 0.1 and 1 μmol/L	(27, 32)
Nigericin	2.5, 5 and 7.5 μmol/L	(27, 33)
Nonactin	0.1, 1 and 10 μmol/L	(26, 27)
Nystatin	1, 10, 20, 30 and 100 μmol/L	(29)
Valinomycin	0.001, 0.01 and 0.1 μmol/L	(2, 4, 26, 32, 33)

propagated under standard tissue culture conditions, grown as monolayer culture in full medium; Eagle's minimal essential medium (MEM) with Earl's salts supplemented with 10% foetal calf serum and 200 μmol/L L-glutamine. They were incubated at 37˚C in a humidified atmosphere containing 5% CO_2.

Efflux and influx K^+ blockers at given concentrations were chosen to represent the most frequently expressed K^+ channels. 4-Aminopyridine (4-AP, 5 mmol/L), charybdotoxin (4.8 μmol/L), iberiotoxin (0.01 μmol/L), margatoxin (0.05 μmol/L) and quinidine (50 μmol/L), were used at concentrations chosen from other studies, but higher concentrations were also used to rule out the possibility of undersized concentrations (15-22). The concentration of ouabain (10 μmol/L) together with bumetanide (10 μmol/L) and tetraethyl ammonium (TEA, 1 mmol/L) were chosen as their effects on the P31 cell line have previously been elucidated (10).

Dose responses of the ionophores amphotericin B, beauvericin, digitonin, gramicidin D, nigericin, nonactin, nystatin and valinomycin (Table I) were tested. Their respective concentrations were chosen based on demonstrated ionophore effects on cellular K^+ efflux, permeabilisation of the cell membrane and/or induction of apoptosis in cell systems (2, 4, 23-35).

For the $^{86}Rb^+$ efflux assay, P31 cells (25,000 cells/well) were plated into 96-well culture plates (Isoplate, Perkin Elmer Wallac, Turku, Finland) and maintained in full medium overnight (at 37˚C in a humidified atmosphere containing 5% CO_2). On the following day, $^{86}Rb^+$ was added (0.75 mCi/L in full medium) and the cells were again incubated overnight. After aspiration of the media, 100 μL of medium, with or without test substances, were added to the wells. At the end of each time-interval, the supernatant was aspirated and transferred to 96-well plates (Flexible plate, Perkin Elmer Wallac). After addition of 150 μL Optiphase SuperMix Liquid Scintillation Cocktail (Perkin Elmer Wallac), the $^{86}Rb^+$ content of the cells and the supernatants was counted using a MikroBeta counter (Perkin Elmer Wallac). The same protocol was followed for U1690 cells, but 40,000 cells were plated. The percentage of $^{86}Rb^+$ efflux was calculated in relation to the total cellular content of $^{86}Rb^+$, according to following formula:

$$\% \text{ efflux} = 100 \times \text{efflux} / (\text{efflux} + \text{remain})$$

where "efflux" is the counts-per-minute (cpm)-value in the efflux solution and "remain" is the cpm-value of $^{86}Rb^+$ remaining in the cells. The experiments were repeated 8-14 times.

For $^{86}Rb^+$ influx assay, P31 cells (25,000 cells/well) were plated into 96-well culture plates and maintained in full medium for 48 h at 37˚C in a humidified atmosphere containing 5% CO_2. After aspiration of the media, 100 μL of full medium, with or without the test substances, alone or in combination and with $^{86}Rb^+$ (0.75 mCi/L), were added to the wells. After each time-interval, the supernatants were aspirated and the cells washed twice with 200 μL NaCl. The Liquid Scintillation Cocktail (150 μL) was added and $^{86}Rb^+$ was counted by using a MikroBeta counter. The same protocol was used for U1690 cells, but 40,000 cells were plated. The result of $^{86}Rb^+$ influx is expressed as the percentage of untreated control cells for each time-point. The experiments were repeated 8-24 times (6-24 for control).

A lactate dehydrogenase (LDH) release test (CytoTox 96®, Promega Corporation, Madison, USA) was used to determine cytotoxicity. Released LDH in the culture supernatant forms a red formazan product of a tetrazolium salt. The assay was carried out according to the manufacturer's instructions. P31 cells (15,000 cells/well) were plated into 96-well culture plates and maintained in full medium overnight, incubated at 37˚C in a humidified atmosphere containing 5% CO_2. On the following day, the medium was replaced with medium with 3 or 6 mg/L amphotericin B, 5 or 7.5 μmol/L nigericin, or 10 μmol/L bumetanide in combination with 10 μmol/L ouabain. Control wells without test substances were used as background. Eight wells were used to set maximum LDH release (100%). Absorbance at 490 nm was determined in a microplate reader (Molecular Devices, USA). The same protocol was used for U1690 cells, but 40,000 cells were plated. The results were presented as the percentage of maximum LDH release with background absorbance deducted.

4-Aminopyridine (4-AP, CAS 504-24-5), amphotericin B (CAS 1397-89-3), beauvericin (CAS 26048-05-5), bumetanide (CAS 28395-03-01), charybdotoxin (dissolved in 100 μL NaCl and stored at 20˚C) (CAS 95751-30-7), digitonin (CAS 11024-24-1), gramicidin D (CAS 1405-97-6), iberiotoxin (dissolved in 100 μL NaCl and stored at 20˚C) (CAS 129203-60-7), margatoxin (dissolved in 100 μL NaCl and stored at 20˚C) (CAS 145808-47-5), nigericin (CAS 28380-24-7), nonactin (CAS 6833-87-7), nystatin (CAS 1400-61-9), ouabain (CAS 11018-89-6), quinidine (CAS 56-54-2), tetraethyl ammonium (TEA, CAS 56-34-8) and valinomycin (CAS 2001-95-8) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). $^{86}Rb^+$ (CAS 7791-11-9) was purchased from Amersham International (Amersham, UK). The culture medium (Eagle's MEM with Earle's salts) and L-glutamine were from Gibco Ltd. (Paisley, Scotland, UK). Foetal calf serum was from Biochrom KG (Berlin, Germany). All other chemicals were of analytical grade.

The statistical significance of the differences between experiments was tested with one-way ANOVA. The level of significance for rejecting the null hypothesis of zero treatment effect was $p=0.05$.

Results

The efflux of $^{86}Rb^+$ of control or test cells was not linear over time as the initial efflux at time 0 with this methodology was momentary and reached (treated or untreated cells) 12-17% in P31 cells (Figure 1A and 1B) and 20-24% of total $^{86}Rb^+$ in U1690 cells (Figure 2A). At 6 h, the control $^{86}Rb^+$ efflux reached 89-90% (Figure 1A and 1B) in P31 cells and 87% (Figure 2A) in U1690 cells. Thus, after the initial rapid efflux, the rate of $^{86}Rb^+$ efflux under

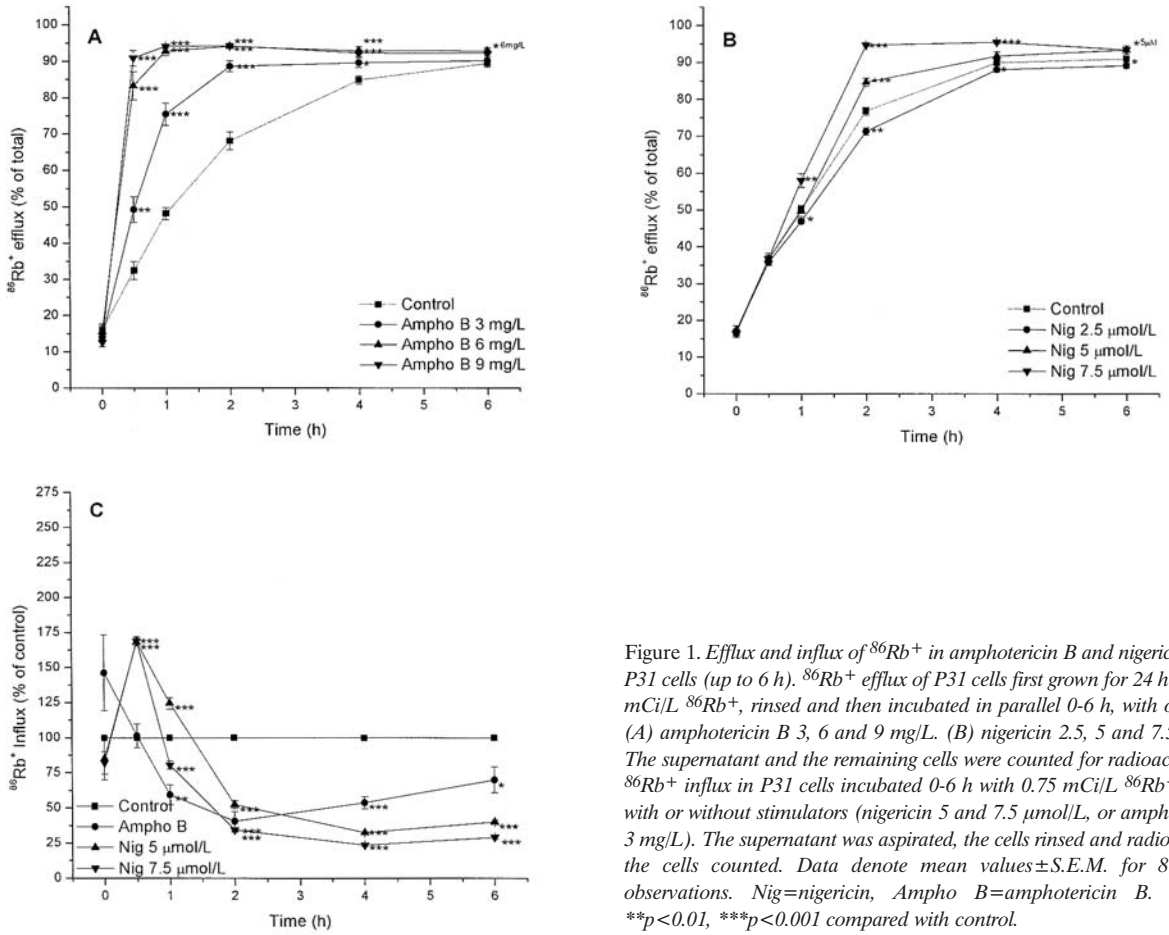

Figure 1. *Efflux and influx of $^{86}Rb^+$ in amphotericin B and nigericin-treated P31 cells (up to 6 h). $^{86}Rb^+$ efflux of P31 cells first grown for 24 h with 0.75 mCi/L $^{86}Rb^+$, rinsed and then incubated in parallel 0-6 h, with or without (A) amphotericin B 3, 6 and 9 mg/L. (B) nigericin 2.5, 5 and 7.5 μmol/L. The supernatant and the remaining cells were counted for radioactivity. (C) $^{86}Rb^+$ influx in P31 cells incubated 0-6 h with 0.75 mCi/L $^{86}Rb^+$ together with or without stimulators (nigericin 5 and 7.5 μmol/L, or amphotericin B 3 mg/L). The supernatant was aspirated, the cells rinsed and radioactivity in the cells counted. Data denote mean values±S.E.M. for 8 separate observations. Nig=nigericin, Ampho B=amphotericin B. *p<0.05, **p<0.01, ***p<0.001 compared with control.*

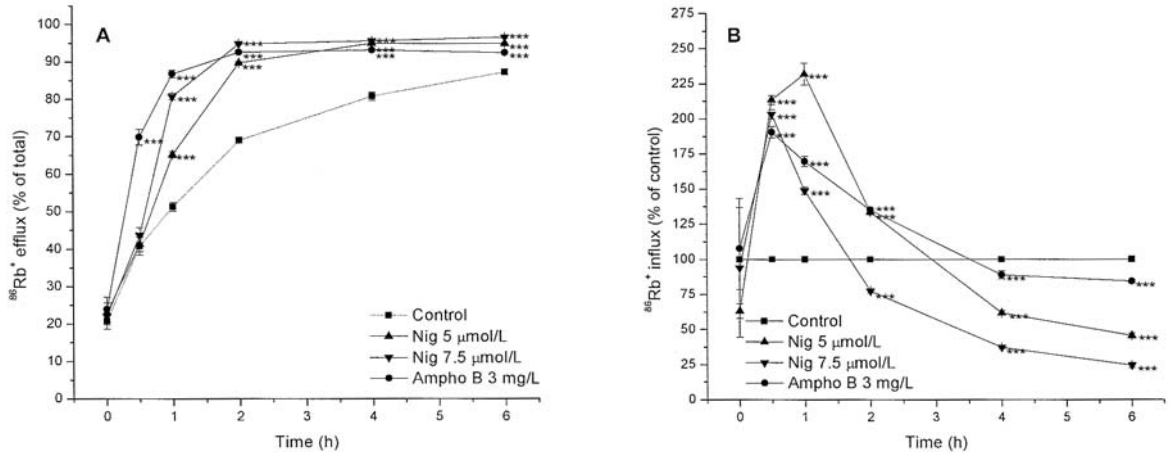

Figure 2. *Efflux and influx of $^{86}Rb^+$ in amphotericin B and nigericin-treated U1690 cells (up to 6 h). (A) $^{86}Rb^+$ efflux in U1690 cells first grown for 24 h with 0.75 mCi/L $^{86}Rb^+$, rinsed and then incubated in parallel 0-6 h, without or with nigericin 5 and 7.5 μmol/L, or amphotericin B 3 mg/L. The supernatant and the remaining cells were counted for radioactivity. (B) $^{86}Rb^+$ influx in U1690 cells incubated 0 - 6 h with 0.75 mCi/L $^{86}Rb^+$ together with or without nigericin 5 and 7.5 μmol/L, or amphotericin B 3 mg/L. The supernatant was aspirated, the cells rinsed and radioactivity in the cells counted. Nig=nigericin, Ampho B=amphotericin B. Data denote mean values±S.E.M. of $^{86}Rb^+$ influx in untreated control for 8 separate observations. ***p<0.001.*

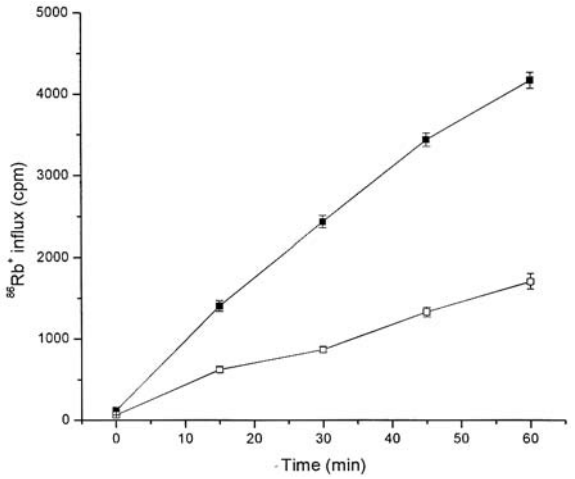

Figure 3. *Basal $^{86}Rb^+$ influx in P31 and U1690 cells. $^{86}Rb^+$ influx in P31 (■) and U1690 cells (□) incubated 0 - 60 min with 0.75 mCi/L $^{86}Rb^+$. The supernatant was aspirated, the cells were rinsed and radioactivity in the cells counted. Data denote mean values±S.E.M. for 28-68 separate observations.*

control conditions was similar for both cell lines. The influx of $^{86}Rb^+$ in both U1690 and P31 cells was non-linear for the first 60 min. The U1690 cells showed less than half the uptake rate of P31 cells (Figure 3). The U1690 cells are smaller and might therefore contain fewer K^+ channels or pumps; alternatively, the K^+ uptake activity is reduced in the U1690 cells.

To elucidate the possibility of pharmacologically controlling the efflux of Rb^+, we tested inhibitors of some of the most frequently expressed potassium ion channels. As a screening, we first tested a set of inhibitors up to 30 min. In the P31 cell line, iberiotoxin (0.01 μmol/L) had an inhibitory effect, whereas charybdotoxin (4.8 μmol/L) and the other efflux inhibitors did not (results not shown). This suggested the presence of BK channels in the human pulmonary mesothelioma cell line. However, on repeating the experiment up to 60 min, we noted no significant effect of iberiotoxin alone (results not shown). The results in this study confirm previous results (10), demonstrating Na^+, K^+, ATPase pump and Na^+, K^+, $2Cl^-$-cotransport activity as the main mechanisms of K^+ influx, not only for the initial influx, but also for continued influx up to 60 min (results not shown). When testing the efflux inhibitors on U1690 cells, only quinidine had a significant effect when run as a 30-min $^{86}Rb^+$ efflux screening (results not shown), which suggested the TWIK1/TASK2 or hERG type of K^+ channel involvement in K^+ efflux of the cell line. However, on repeating the experiment for a longer period, the agent alone had no effect on $^{86}Rb^+$ efflux (results not shown). As with the P31 cells, bumetanide initially (at 15 min) markedly decreased $^{86}Rb^+$ influx. Thereafter, the influx inhibition gradually decreased to the end of the period. Ouabain (10 μmol/L) had a marked and persistent inhibitory effect on $^{86}Rb^+$ influx. As with P31, the combination of bumetanide and ouabain did not demonstrate an initial additive inhibition of $^{86}Rb^+$ influx, but this gradually developed with time and reached a maximal inhibition of 25% of the untreated control cell values (results not shown).

With the initial experiments, we established that K^+ efflux was less likely to be affected by specific efflux channel inhibitors and, therefore, no stimulation by specific stimulators was anticipated. K^+ influx, however, could be effectively inhibited by the combination of bumetanide and ouabain. To pharmacologically stimulate the efflux of $^{86}Rb^+$, we therefore tested a set of known K^+ ionophores on the P31 cell line (Table I). Of them, only amphotericin B, nigericin and digitonin induced $^{86}Rb^+$ efflux. Digitonin was excluded from further studies since a considerable number of adherent P31 cells were lost during the experiments. Amphotericin B (3, 6 and 9 mg/L) stimulated $^{86}Rb^+$ efflux in all three concentrations (Figure 1A). Earlier studies have shown that 3 mg/L is less toxic than the higher concentrations and 4 mg/L has been suggested as the tolerated maximal serum concentration with acceptable adverse effects (36). With 6 and 9 mg/L amphotericin B, the efflux was initially very rapid (0.5 h), almost immediately emptying the cells of the isotope. The efflux induced by 3 mg/L amphotericin B was less dramatic, but after 1-h incubation the efflux of $^{86}Rb^+$ was two-fold that of control cells (Figure 1A). Nigericin (5 and 7.5 μmol/L) also stimulated efflux (Figure 1B), but much less dramatically, although the higher concentration had almost completely emptied the P31 cells of $^{86}Rb^+$ after 2-h incubation. Nigericin 2.5 μmol/L did not significantly stimulate $^{86}Rb^+$ efflux and was, therefore, excluded from further studies (Figure 1B). When amphotericin B and nigericin were studied on $^{86}Rb^+$ influx, an initial stimulation was noted with both 5 μmol/L and 7.5 μmol/L nigericin, but already at 2 and 1 h, respectively, the $^{86}Rb^+$ influx was markedly inhibited, an inhibition which persisted through the 6-h test period. Amphotericin B initially did not significantly stimulate influx, but, after 1 h, a significant ($p<0.01$) $^{86}Rb^+$ influx inhibition was noted. The inhibitory effect decreased with time, but remained marked throughout the test period (Figure 1C). When tested on the U1690 cell line, nigericin (5 μmol/L and 7.5 μmol/L) as well as amphotericin B (3 mg/L) stimulated $^{86}Rb^+$ efflux (Figure 2A). As with the P31 cells, the efflux induced by nigericin was slower than that of amphotericin B, but both agents had almost entirely emptied the cells of loaded $^{86}Rb^+$ at 2 h. In contrast to the $^{86}Rb^+$ influx reaction of the P31 cells, the U1690 cells reacted with a more marked stimulation of the influx when

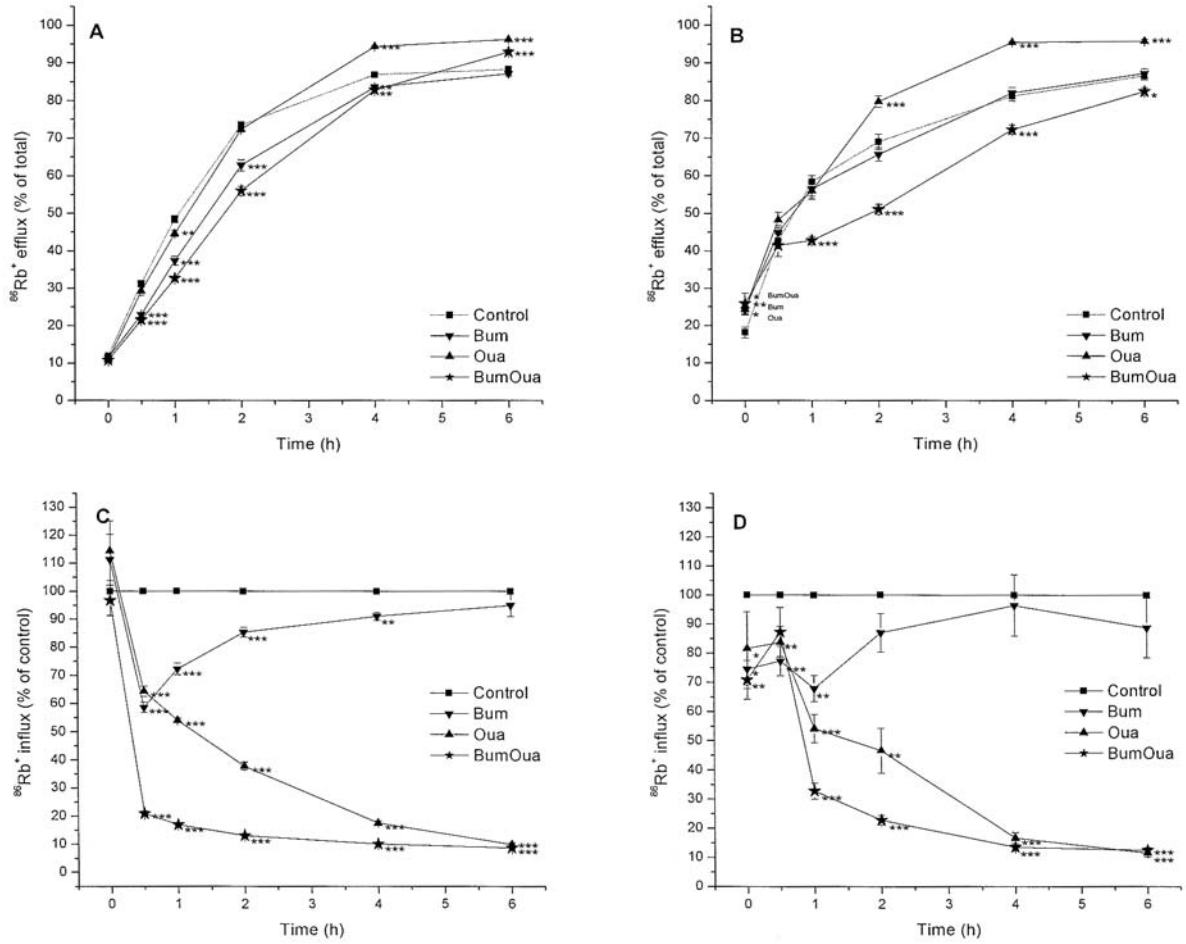

Figure 4. *Efflux and influx of $^{86}Rb^+$ in P31 and U1690 cells treated with bumetanide and ouabain alone or in combination (up to 6 h). $^{86}Rb^+$ efflux in P31 cells (A) and U1690 cells (B) first grown for 24 h with 0.75 mCi/L $^{86}Rb^+$, rinsed and then incubated in parallel 0-6 h, with or without test substances. The supernatant and the remaining cells were counted for radioactivity. $^{86}Rb^+$ influx in P31 cells (C) and U1690 cells (D) incubated 0-6 h with 0.75 mCi/L $^{86}Rb^+$ together with or without inhibitors. The supernatant was aspirated, the cells rinsed, and radioactivity in the cells counted. Bum=10* μmol/*L bumetanide, Oua=10 μmol/L ouabain. Data denote mean values±S.E.M. of $^{86}Rb^+$ influx in untreated control for 8 separate observations. *$p<0.05$, **$p<0.01$, ***$p<0.001$.*

incubated with 5 and 7.5 μmol/L nigericin, as well as 3 mg/L amphotericin B (Figure 2B). The $^{86}Rb^+$ influx increase was also more prolonged before turning to an inhibition. The influx inhibition of amphotericin B was small compared to the inhibition seen with the P31 cells at later stages of the incubation period (Figure 2B). The activity of the K^+ influx inhibitors bumetanide (10 μmol/L) or ouabain (10 μmol/L) alone, and in combination were also tested for up to 6 h to see if the effects noted during the short-term incubations were lasting. Bumetanide alone inhibited the $^{86}Rb^+$efflux in P31 cells on incubation for up to 4 h, whereas ouabain alone had no effect on the efflux compared to the control cells for the first 2 h. At 4- and 6-h incubation, ouabain alone stimulated the $^{86}Rb^+$efflux (Figure 4A). When bumetanide and ouabain were combined, an inhibition of the efflux was noted for incubation up to 4 h, but at 6 h a stimulating effect was seen. Bumetanide alone transiently inhibited P31 $^{86}Rb^+$ influx from a marked inhibition at 0.5-h incubation to non-significant compared to control at 6 h incubation (Figure 4C). Ouabain alone effectively inhibited the $^{86}Rb^+$influx during the 6-h test period and, when combined with bumetanide, the $^{86}Rb^+$ influx was additive throughout the 6-h period (Figure 4C). Ouabain stimulated the U1690 cell $^{86}Rb^+$ efflux also after 2, 4 and 6 h (Figure 4B). No effect was seen with bumetanide alone, but, when combined with ouabain, the $^{86}Rb^+$ efflux was markedly inhibited from 1 h and throughout the 6-h test period (Figure 4B). The influx of $^{86}Rb^+$ in U1690 cells (Figure 4D) was similar to P31 cells (Figure 4C). Bumetanide alone had a significant ($p<0.01$) $^{86}Rb^+$ influx effect up to 1 h, after which the

effect decreased and was not different from the control. Bumetanide combined with ouabain had an almost additive inhibition of the $^{86}Rb^+$ influx on U1690 cells (Figure 4D).

LDH release was used to test the cytotoxicity of the efflux stimulators and influx inhibitors. In P31 cells, 3 mg/L amphotericin B induced a small LDH release (1.52±0.92% and 4.02±0.85% at 3 and 6 h, respectively). An accentuated (10.9±1.41%) LDH release was noted with 6 mg/L amphotericin B after 3-h incubation, increasing to 26.4±1.31% after 6 h. With nigericin 5 and 7.5 μmol/L, –2.13±1.30% at 3 h and 3.78±1.61% after 6 h for the low concentration, and 4.10±0.46% and 2.70±1.42% at 3 and 6 h for the higher concentration were noted. On combining bumetanide 10 μmol/L and ouabain 10 μmol/L, no LDH release was shown at 3 h (–7.09±3.02% or 6 h –0.78±1.15%), respectively. In U1690 cells, amphotericin B 3 mg/L induced no LDH release (0.74±0.64%) at 3 h or 6 h (–1.20±1.25%), whereas 6 mg/L amphotericin B induced 14.0±1.40% and 54.7±1.38% LDH release at 3 h and 6 h, respectively. Nigericin 5 and 7.5 μmol/L induced a small LDH release (5.13±2.24% and 8.7±2.09%, respectively) at 3 h, but high (63.6±2.86% and 73.6±2.63%) LDH release at 6 h. As in the P31 cells, there was no cytotoxic effect of the combination bumetanide / ouabain with an LDH release of –4.57±1.37% at 3 h and –5.27±1.30% at 6 h.

Discussion

A major enhancement of cellular $^{86}Rb^+$ efflux was demonstrated by the ionophores amphotericin B, nigericin and digitonin, although the latter two were cytotoxic. When bumetanide was combined with the Na^+, K^+, ATPase pump inhibitor ouabain, a marked and lasting (tested up to 6 h) $^{86}Rb^+$ influx inhibition was noted in both cell lines, however, a considerable efflux inhibition was also noted on the U1690 cells. Thus, pharmacologically persistent K^+ influx inhibition and simultaneous K^+ efflux stimulation of cancer cells can reduce intracellular K^+ and may enhance apoptosis during cancer treatment.

Few studies on the effects of pharmacological drugs on K^+ channel activity in intact adherent tumour cells have been performed. The cell growth of an androgen-sensitive prostate cancer cell line was inhibited by K^+ flux inhibitors, indicating the role of K^+ channels in tumour progression (37). K^+ channel blockers caused growth inhibition of four colon cancer cell lines expressing voltage-gated K^+ channels, suggesting that K^+ channels may be important therapeutic targets (38). They also demonstrated significant growth-inhibition of K^+ blockers on breast cancer cells (39). Our previous studies on the pulmonary mesothelioma cell line P31 showed that Na^+, K^+, ATPase and Na^+, K^+, $2Cl^-$-cotransport are responsible for almost all of the influx of K^+ (10), whereas no characterization of the efflux mechanisms of the cells has hitherto been performed. We now expanded the studies to include the small cell cancer cell line U1690 as lung cancers generally have poor prognosis and treatment modalities still include well-known apoptosis inducers such as cisplatin and radiation. The ionophore amphotericin B produces pores in the membrane (40) and increases the opening probability of K^+ channels by a factor of six (41). Combined with the Na^+, K^+, $2Cl^-$-cotransport blockers bumetanide, amphotericin B augments cisplatin-induced apoptosis, observed as increased nucleosome formation and caspase-3 activity of mesothelioma cells (42).

By using $^{86}Rb^+$ (K^+ analogue), we studied the influx and efflux of K^+ in the human pulmonary mesothelioma (P31) and small cell lung cancer (U1690) cell lines. The K^+ flux inhibitors were chosen from their known effects on K^+ channels, whether selective or not. Some of them, such as 4-aminopyridine (16, 18) and quinidine (22), are also known to affect cell proliferation (18). There were, however, only minute effects of the K^+ flux inhibitors on $^{86}Rb^+$ efflux, suggesting that channel-specific inhibition of the K^+ efflux is not easily obtained in these cell lines. We instead chose to test a number of ionophores, which induce efflux of K^+ down its concentration gradient. Amphotericin B (43), but also other ionophores such as beauvericin (28, 32), nystatin (30) and valinomycin (4, 23, 26, 29) have been shown to induce or enhance apoptosis.

Of the tested ionophores, amphotericin B, digitonin and nigericin stimulated $^{86}Rb^+$ efflux in both cell lines, however, digitonin was excluded from further experiments due to the loss of cell adhesion during treatment. Nigericin at 5 and 7.5 μmol/L was shown to be cytotoxic in U1690 cells at 6 h, but not in P31 cells. A high concentration (6 mg/L) of amphotericin B was toxic in U1690 cells at 6 h, but with less cytotoxic effect in P31 cells. Of note is also that the ionophores induced a short-term stimulation of the $^{86}Rb^+$ influx, followed later by an often marked and prolonged influx inhibition. This could be due to an initial cellular compensatory influx response for the ionophore-induced K^+ efflux, but the following influx inhibition suggests that at least some ionophores such as (seen here) nigericin *per se* could deprive cells of K^+, not only by efficient stimulation of efflux, but also by reduction of a compensatory K^+ influx response.

We found that the K^+ influx in both P31 and U1690 cells is, to a large extent, controlled by Na^+, K^+, $2Cl^-$-cotransport and Na^+, K^+, ATPase pump activity. The Na^+, K^+, $2Cl^-$-cotransport inhibitor bumetanide markedly inhibited the K^+ influx in both cell lines up to 60 min, however the inhibition was transient and did not remain significantly different from the control after 6 h. Bumetanide, in combination with the Na^+, K^+, ATPase pump inhibitor ouabain, had an additive inhibitory effect in the P31 cell line for all time-points measured, whereas the additive effects in the U1690 cell line were noted later. Bumetanide also inhibited the $^{86}Rb^+$ efflux

of P31 cells, whereas no effect was seen on U1690 cells. The combination of bumetanide with ouabain also inhibited the $^{86}Rb^+$ efflux, especially in the U1690 cells. The overall effect on intracellular potassium ion balance of bumetanide *per se*, or when combined with ouabain, therefore, seems difficult to predict with $^{86}Rb^+$ flux methodology. Other methods that directly quantify the intracellular K^+ concentration have to be used. The combination of bumetanide and ouabain resulted in a rapid and marked inhibition of the $^{86}Rb^+$ influx, suggesting that less pronounced and slowly emerging K^+ inhibition is possible to achieve by the drugs alone or in combination, depending on the applied concentration of the drugs.

This is the first study of the pharmacological effects on U1690 cells with respect to K^+ fluxes. The small cell pulmonary cancer cell line shares similar characteristics with the P31 line regarding the influx of potassium ions. Na^+, K^+, $2Cl^-$-cotransport and Na^+, K^+, ATPase pump activity are the main mechanisms controlling the influx of potassium ions. The effect of inhibitors on the K^+ efflux channels was small, except for bumetanide on P31 cells. The differences noted demonstrate the importance of describing the pharmacological effects on K^+ fluxes before attempting to manipulate intracellular K^+ concentrations for therapeutic purposes. Probably, intracellular K^+ homeostasis will not be disrupted as long as the K^+ efflux can be counterbalanced by a sufficient K^+ uptake. Since Na^+, K^+, ATPase and Na^+, K^+, $2Cl^-$-cotransport activity are the dominating mechanism for K^+ uptake, their activity is probably an important factor in controlling K^+ homeostasis and induction of apoptosis in the tested cell lines. From our results, we conclude that the influx of K^+ can be efficiently inhibited by Na^+, K^+, ATPase and/or Na^+, K^+, $2Cl^-$-cotransport inhibition, and that the efflux can be stimulated using ionophores. In future studies aiming to deprive cells of K^+, pharmacological manipulation of the K^+ flux mechanisms would seem to be effective enough to provoke or enhance apoptosis, which could be used to improve the efficacy of traditionally used approaches in cancer treatment.

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References

1 Bortner CD, Hughes FM Jr and Cidlowski JA: A primary role for K^+ and Na^+ efflux in the activation of apoptosis. J Biol Chem *272*: 32436-32442, 1997.

2 Krick S, Platoshyn O, Sweeney M, Kim H and Yuan JX: Activation of K^+ channels induces apoptosis in vascular smooth muscle cells. Am J Physiol Cell Physiol *280*: C970-C979, 2001.

3 Yu SP, Yeh CH and Strasser V: NMDA receptor-mediated K^+ efflux and neuronal apoptosis. Science *284*: 336-339, 1999.

4 Yu SP, Yeh CH, Sensi SL, Gwag BJ, Canzoniero LM, Farhangrazi ZS, Ying HS, Tian M, Dugan LL and Choi DW: Mediation of neuronal apoptosis by enhancement of outward potassium current. Science *278*: 114-117, 1997.

5 Wang X, Xiao AY, Ichinose T and Yu SP: Effects of tetraethylammonium analogs on apoptosis and membrane currents in cultured cortical neurons. J Pharmacol Exp Ther *295*: 524-530, 2000.

6 Greger R, Heitzmann D, Hug MJ, Hoffmann EK and Bleich M: The Na^+ K^+ $2Cl^-$ cotransporter in the rectal gland of *Squalus acanthias* is activated by cell shrinkage. Pflugers Arch Eur J Physiol *438*: 165-176, 1999.

7 Hoffman EK and Dunham PB: Membrane mechanisms and intracellular signalling in cell volume regulation. Int Rev Cytol *161*: 173-262, 1995.

8 Lytle C: Activation of the avian erythrocyte Na^+ K^+ $2Cl^-$ cotransport protein by cell shrinkage, cAMP, fluoride, and calyculin-A involves phosphorylation at common sites. J Biol Chem *272*: 15069-15077, 1997.

9 Lang F, Busch GL, Ritter M, Volkl H, Waldegger S, Gulbins E and Häussinger D: Functional significance of cell volume regulatory mechanisms. Physiol Rev *78*: 247-306, 1998.

10 Sandström P-E, Jonsson Ö, Grankvist K and Henriksson R: Identification of potassium flux pathways and their role in the cytotoxicity of estramustine in human malignant glioma, prostatic carcinoma and pulmonary carcinoma cell lines. Eur J Cancer *30A*: 1822-1826, 1994.

11 Marklund L, Andersson B, Behnam-Motlagh P, Sandström P-E, Henriksson R and Grankvist K: Cellular potassium ion deprivation enhances apoptosis induced by cisplatin. Basic Clin Pharmacol Toxicol *94*: 245-251, 2004.

12 Cheng CS, Alderman D, Kwash J, Dessaint J, Patel R, Lescoe MK, Kinrade MB and Yu W: A high-throughput HERG potassium channel function assay: an old assay with a new look. Drug Dev Ind Pharm *28*: 177-191, 2002.

13 Marklund SL, Westman GN, Lundgren E and Roos G: Copper- and zinc-containing superoxide dismutase, manganese-containing superoxide dismutase, catalase, and glutathione peroxidase in normal and neoplastic human cell lines and normal human tissues. Cancer Res *42*: 1955-1961, 1982.

14 Bergh J, Nilsson K, Ekman R and Giovanella B: Establishment and characterization of cell lines from human small cell and large cell carcinomas of the lung. Acta Pathol Microbiol Immunol Scand Sect *A93*: 133-147, 1985.

15 Calderone V: Large-conductance Ca^{2+}-activated K^+ channels: function, pharmacology and drugs. Curr Med Chem *9*: 1385-1395, 2002.

16 Chin LS, Park CC, Zitnay KM, Sinha M, DiPatri AJ Jr, Perillan P and Simard JM: 4-Aminopyridine causes apoptosis and blocks an outward rectifier K^+ channel in malignant astrocytoma cell lines. Neurosci Res *48*: 122-127, 1997.

17 Felix JP, Bugianesi RM, Schmalhofer WA, Borris R, Goetz MA, Hensens OD, Bao JM, Kayser F, Parsons WH, Rupprecht K, Garcia ML, Kaczorowski GJ and Slaughter RS: Identification and biochemical characterization of a novel nortriterpene inhibition of the human lymphocyte voltage-gated potassium channel Kvl.3. Biochemistry *38*: 4922-4930, 1999.

18 Fraser SP, Grimes JA and Djamgoz MB: Effects of voltage-ion channel modulators on rat prostatic cancer cell proliferation: comparison of strongly and weakly metastatic cell lines. Prostate *44*: 61-76, 2000.

19 Kim JH, Hong EK, Choi HS, Oh SJ, Kim KM, Uhm DY and Kim SJ: K^+ channel currents in rat ventral prostate epithelial cells. Prostate *51*: 201-210, 2002.

20 Shieh CC, Coghlan M, Sullivan JP and Gopalakrishnan M: Potassium channels: molecular defects, diseases, and therapeutic opportunities. Pharmacol Rev *52*: 557-593, 2000.

21 Tahara S, Fukuda K, Kodama H, Kato T, Miyoshi S and Ogava S: Potassium channel blocker activates extracellular signal-regulated kinases through Pyk2 and epidermal growth factor receptor in rat cardiomyocytes. J Am Coll Cardiol *38*: 1554-1563, 2001.

22 Utermark T, Alekov A, Lerche H, Abramowski V, Giovannini M and Hanemann CO: Quinidine impairs proliferation of neurofibromatosis type 2-deficient human malignant mesothelioma cells. Cancer *97*: 1955-1962, 2003.

23 Allbritton NL, Verret CR, Wolley RC and Eisen HN: Calcium ion concentrations and DNA fragmentation in target cell destruction by murine cloned cytotoxic T lymphocytes. J Exp Med *167*: 514-527, 1988.

24 Calo L, Fornelli F, Ramires R, Nenna S, Tursi A, Caiaffa MF and Macchia L: Cytotoxic effects of the mycotoxin beauvericin to human cell lines of myeloid origin. Pharmacol Res *49*: 73-77, 2004.

25 Gottlieb RA and Granville DJ: Analyzing mitochondrial changes during apoptosis. Methods *26*: 341-347, 2002.

26 Deckers CLP, Lyons AB, Samuel K, Sanderson A and Maddy AH: Alternative pathways of apoptosis induced by methylprednisolone and valinomycin analyzed by flow cytometry. Exp Cell Res *208*: 362-370, 1993.

27 DoebIer JA: Effects of neutral ionophores on membrane electrical characteristics of NG1O8-15 cells. Toxicol Lett *114*: 27-38, 2000.

28 Harnois DM, Que FG, Celli A, LaRusso NF and Gores GJ: Bcl-2 is overexpressed and alters the threshold for apoptosis in a cholangiocarcinoma cell line. Hepatology *26*: 884-890, 1997.

29 Lacour S, Hammann A, Grazide S, Lagadic-Gossmann D, Athias A, Sergent O, Laurent G, Gambert P, Solary E and Dimanche-Boitrel MT: Cisplatin-induced CD95 redistribution into membrane lipid rafts of HT29 human colon cancer cells. Cancer Res *64*: 3593-3598, 2004.

30 Maeno E, Ishizaki Y, Kanaseki T, Hazama A and Okada Y: Normotonic cell shrinkage because of disordered volume regulation is an early prerequisite to apoptosis. Proc Natl Acad Sci USA *97*: 9487-9492, 2000.

31 Nash PB, Purner MB, Leon RP, Clarke P, Duke RC and Curiel TJ: Toxoplasma gondii-infected cells are resistant to multiple inducers of apoptosis. J Immunol *160*: 1824-1830, 1998.

32 Pe1assy C, Breittmayer JP and Aussel C: Regulation of phosphatidylserine exposure at the cell surface by the serine-base exchange enzyme system during CD95-induced apoptosis. Biochem Pharmacol *59*: 855-863, 2000.

33 Penning LC, Denecker G, Vercammen D, Declercq W, Schipper RG and Vandenabee1e P: A role for potassium in TNF-induced apoptosis and gene-induction in human and rodent tumour cell lines. Cytokine *12*: 747-750, 2000.

34 Segal R and Milo-Goldzweig I: The susceptiblility of cholesterol-depleted erythrocytes to sapinin and sapogen hemolysis. Biochim Biophys Acta *512*: 223-226, 1978.

35 Vercesi AE, Bernardes CF, Hoffmann ME, Gadelha FR and Docampo R: Digitonin permeabilization does not affect mitochondrial function and allows the determination of the mitochondrial membrane potential of *Trypanosoma cruzi in situ*. J Biol Chem *266*: 14431-14434, 1991.

36 Atkinson AJ Jr and Bennett JE: Amphotericin B pharmacokinetics in humans. Antimicrob Agents Chemother *13*: 271-276, 1978.

37 Skryma RN, Prevarskaya NB, Dufy-Barbe L, Odessa MF, Audin J and Dufy B: Potassium conductance in the androgen-prostate cancer cell line, LNCaP: involvement in cell proliferation. Prostate *33*: 112-122, 1997.

38 Abdul M and Hoosein N: Voltage-gated potassium ion channels in colon cancer. Oncol Rep *9*: 961-964, 2002.

39 Abdul M, Santo A and Hoosein N: Activity of potassium channel-blockers in breast cancer. Anticancer Res *23*: 3347-3351, 2003.

40 Sharp SY, Mistry P, Valenti MR, Bryant AP and Kelland LR: Selective potentiation of platinum drug cytotoxicity in cisplatin- sensitive and -resistant human ovarian carcinoma cell lines by amphotericin B. Cancer Chemother Pharmacol *35*: 137-143, 1994.

41 Hsu SF and Burnette RR: The effect of amphotericin B on the K-channel activity of MDCK cells. Biochim Biophys Acta *1152*: 189-191, 1993.

42 Marklund L, Henriksson R and Grankvist K: Cisplatin-induced apoptosis of mesothelioma cells is affected by potassium ion flux modulator amphotericin B and bumetanide. Int J Cancer *93*: 577-583, 2000.

43 Marklund L, Henriksson R and Grankvist K: Amphotericin B-apoptosis and cytotoxicity is prevented by the Na^+ K^+ $2Cl^-$ cotransport blocker bumetanide. Life Sci *66*: PL319-324, 2000.

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