Pergamon

European Journal of Cancer Vol. 30A, No. 12, pp. 1822–1826, 1994
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0959-8049/94 $7.00+0.00

0959-8049(94)00236-3

Identification of Potassium Flux Pathways and Their Role in the Cytotoxicity of Estramustine in Human Malignant Glioma, Prostatic Carcinoma and Pulmonary Carcinoma Cell Lines

P.-E. Sandström, Ö. Jonsson, K. Grankvist and R. Henriksson

Clinically-used drugs such as furosemide, bumetanide and cardiac glycosides, are modulators of transmembrane fluxes of cations. Recently, it has been suggested that the regulation of intracellular cation concentrations could be a primary target for anti-neoplastic drugs, and that the cytotoxic activity may be altered by inhibitors of cation fluxes at the level of the plasma membrane. Therefore, we investigated the mechanisms by which cations are translocated across the plasma membrane of malignant glioma (U251 MG), prostatic carcinoma (PC3) and pulmonary carcinoma (P31) cell lines. The interactions between cation flux inhibitors and the cytotoxicity of estramustine were also evaluated. Ouabain, the classical inhibitor of Na^+, K^+ATPase, markedly reduced ^{86}Rb (K^+) influx in all three lines, indicating that this ion transport system is present in the cells. Furosemide and especially bumetanide inhibited the ^{86}Rb influx, indicating the presence of the Na^+, K^+, Cl^- co-transport system. The potassium channel blocker, tetraethylammonium, but not apamin reduced the influx of ^{86}Rb showing that high conductance K^+ channels are present, but that channels of low conductance probably do not exist in these cell lines. The Na^+, K^+, Cl^- co-transport inhibitors furosemide and bumetanide significantly reduced cytotoxicity of estramustine in P31 cells, whereas no interaction between other K^+ flux inhibitors and the anti-neoplastic drugs were detected in any of the cell lines investigated. Thus, the data show that Na^+, K^+, ATPase and Na^+, K^+, Cl^- co-transport systems and K^+ channels of high conductance are present in malignant glioma (U251 MG), prostatic carcinoma (PC3) and pulmonary carcinoma (P31) cell lines, and that inhibition of the Na^+, K^+, Cl^- co-transport system in P31 is associated with reduced cytotoxicity of estramustine. The results justify further studies evaluating the role of these cation flux pathways in terms of targets for anti-neoplastic therapy.

Key words: potassium flux, cytotoxicity, malignant cells, estramustine
Eur J Cancer, Vol. 30A, No. 12, pp. 1822–1826, 1994

INTRODUCTION

FUROSEMIDE, BUMETANIDE and cardiac glycosides are modulators of transmembrane fluxes of cations, and are widely used in the management of diseases such as hypertension as well as heart and renal failure. It is well known that transmembrane fluxes of cations are involved in important cell processes, such as maintenance of the membrane potential [1], regulation of intracellular pH [2] and volume regulation in anisotonic media [2]. The observations that alterations in cation fluxes are involved in mitogen-stimulated cell proliferation [3–5] and that changes in intracellular cation concentrations might be associated with oncogenesis [6] have led to an increased interest in cation transport mechanisms in cancer research.

Subsequently, it has been suggested that cation transport mechanisms can be primary targets for anti-neoplastic drugs [7–9], and that the cytotoxic activity of such drugs is altered by inhibitors of cation transport mechanisms at the level of the plasma membrane [10]. Thus, there exists a potential possibility of interactions between drugs that influence transmembrane fluxes of cations and the specific cancer treatment. Indeed, we found that the clinically-used diuretic amiloride, an inhibitor of Na^+, H^+ exchange, hampers the cytotoxicity of estramustine and bleomycin in transformed fibroblasts [11].

The prostatic cancer cell line PC3, the pulmonary cancer cell line P31 and the malignant human glioma U251 MG are widely used in studies of the cytotoxic mechanisms of anti-neoplastic drugs. However, minor interest has been denoted to the mechanisms of cation transport across their plasma membranes. Therefore, in order to investigate by which mechanisms cations are

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Revised 21 Mar. 1994; accepted 11 Apr. 1994.

translocated across the plasma membrane in these cells, studies on the effects of inhibitors of Na^+, K^+ATPase; Na^+, K^+, Cl^- co-transport and K^+ channels on the influx of $^{86}Rb^+$ (K^+ analogue) were performed. In addition, the effects of potassium flux inhibitors were evaluated on the cytotoxicity of estramustine, which have earlier been suggested to affect the membrane-coupled $^{86}Rb^+$ fluxes [12].

MATERIALS AND METHODS

Cell culture

The three human cell lines PC3 (prostatic cancer), P31 (pulmonary carcinoma) and U251 MG (malignant glioma) were grown as monolayer cultures in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum. The cells were incubated at 37°C in humidified air containing 5% CO_2. The procedure for the growth experiments has been described previously [13, 14]. Briefly, the cells were incubated with or without the test drugs for 6 days. The cell growth was analysed after 3 and 6 days with a Coulter counter multisizer. Each experiment was replicated nine times.

$^{86}Rb^+$ influx

The cells were grown for 30 min in Eagle's MEM, with or without test substances, and then incubated for 5–15 min in the same medium supplemented with 28 μM $^{86}RbCl$. The cells were rinsed, trypsinised and transferred to scintillation vials, and radioactivity was determined by liquid scintillation counting. Isotope uptake in drug-treated cells was expressed as a percentage of the uptake in controls (basal influx) handled in parallel and without test substances.

Chemicals

Furosemide was a gift from Svenska Hoechst AB (Stockholm, Sweden). Ouabain, bumetanide, apamin and tetraethylammonium were obtained from Sigma Chemical Company (St. Louis, Missouri, U.S.A.). Eagle's minimal essential medium was obtained from Gibco (Paisley, U.K.). Fetal calf serum was purchased from Biochrom BG (Berlin, Germany) and $^{86}RbCl$ was purchased from Amersham International (Amersham, U.K.).

RESULTS

The $^{86}Rb^+$ uptake in U251 MG, PC3 and P31 was approximately linear for 15 min (Figure 1). Therefore, it was assumed that the uptake during this time period reflects the influx rate of $^{86}Rb^+$ and is not affected by intracellular accumulation of the isotope.

Effect of ouabain on $^{86}Rb^+$ influx

Ouabain, the classical inhibitor of Na^+, K^+ATPase, markedly reduced $^{86}Rb^+$ influx in all three cell lines (Figure 2). Half-maximum inhibition was observed with 0.1–1 μM ouabain and maximum inhibition was observed with 10 μM of the drug.

Effect of bumetanide and furosemide on $^{86}Rb^+$ influx

Furosemide (0.1–1 mM) inhibited approximately 30% of the 15-min $^{86}Rb^+$ influx in P31 (Figure 3a) and approximately 15% of the influx in PC3 (Figure 3b) and U251 MG (Figure 3c), respectively. The drug concentration for half-maximum inhibition (IC_{50}) was approximately 10 μM. The other loop diuretic, bumetanide, inhibited $^{86}Rb^+$ influx in P31 (Figure 3a), PC3 (Figure 3b) and U251 MG (Figure 3c) to the same extent as furosemide but proved to be about one order of magnitude more potent ($IC_{50} \approx 0.1$–1 μM). Moreover, 100 μM bumetanide also inhibited $^{86}Rb^+$ influx in the presence of the Na^+, K^+ATPase inhibitor ouabain (Figure 4) in all cell lines.

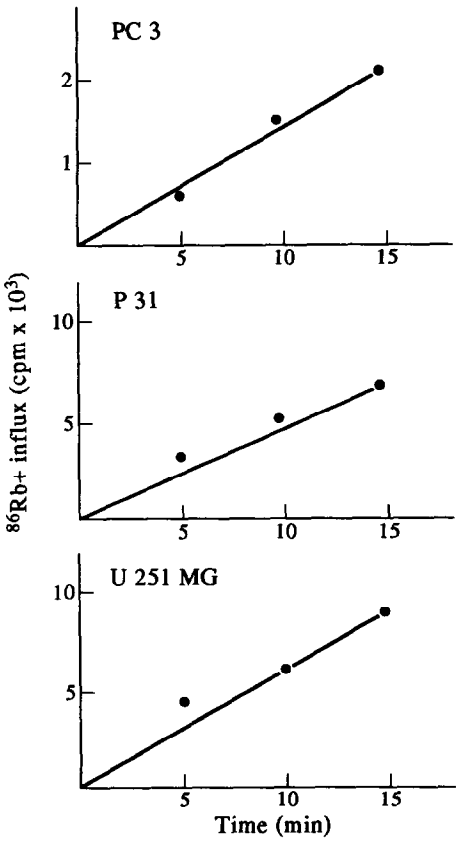

Figure 1. Time-course of $^{86}Rb^+$ influx in PC3, P31 and U251 MG. After 30 min incubation in Eagle's MEM with 10% fetal calf serum, the cells were incubated for 5–15 min with the same medium supplemented with 28 μM $^{86}RbCl$.

Effect of K^+ channels blockers on $^{86}Rb^+$ influx

Tetraethylammonium at 0.1–10 mM reduced $^{86}Rb^+$ influx in U251 MG, PC3 and P31 by approximately 10–15% (Table 1). Since tetraethylammonium is considered to be an inhibitor of K^+ channels with high conductance, it seems likely that this kind of channel is present in these cell lines. Apamin, a bee venom which inhibits a K^+ channel with low conductance, was without effect on $^{86}Rb^+$ influx in the cell lines at concentrations from 1 to 100 nM (data not shown). The data suggest that K^+ channels of high conductance but not of low conductance are present in U251 MG, PC3 and P31.

Effects of potassium flux modulators on cytotoxicity of estramustine

Exposure of the cell lines to estramustine caused a dose-dependent inhibition of the cell growth as has also earlier been described. Ouabain (100 μM) strongly reduced the growth of U251 MG, PC3 and P31 (data not shown). When furosemide and especially bumetanide (Figure 5) were added, the cytotoxicity of estramustine was significantly reduced in the P31 cell line, whereas the effects in the other cells were unaffected. None of the potassium flux modulators alone displayed any signs of intrinsic cytotoxicity (Figure 5).

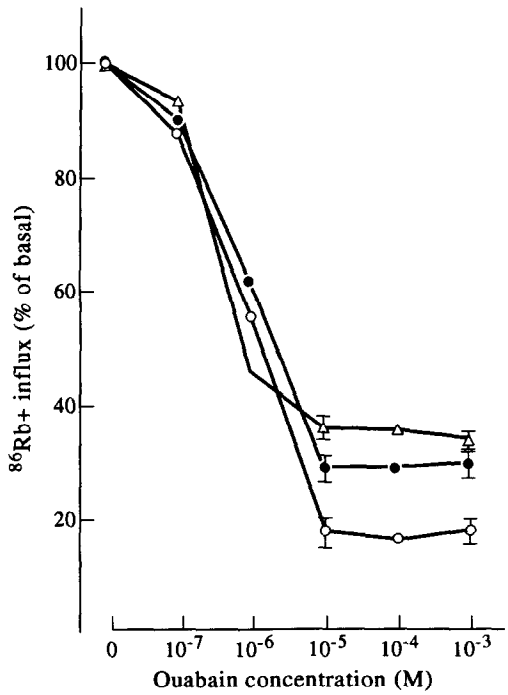

Figure 2. Effect of ouabain on $^{86}Rb^+$ influx in PC3, P31 and U251 MG. After 30 min incubation in Eagle's MEM with 10% fetal calf serum and with or without ouabain, the cells were incubated for 15 min with the same medium supplemented with 28 μm ^{86}RbCl. Data are expressed as mean values ± S.E.M. (n = 6). △ = P31, • = PC3, ○ = MG251. No S.E. bars means variation smaller than the symbol.

DISCUSSION

The present study displayed that at least three different potassium flux pathways exist in the tumour cell lines analysed (malignant glioma U251 MG, prostatic carcinoma PC3 and lung adenocarcinoma P31), i.e. Na^+, K^+ATPase, Na^+, K^+, Cl^- co-transport system and K^+ channels with high conductance. In the cell line (P31) with the most pronounced Na^+, K^+, Cl^- co-transport activity, the cytotoxic effect of estramustine was reduced by inhibition of this transport system with furosemide and bumetanide. Thus, the results indicate that the co-transport system for chloride and cations in the plasma membrane [15–17] is of special importance, at least with regard to the cytotoxicity of estramustine in this experimental situation.

The reduced $^{86}Rb^+$ influx caused by ouabain strongly suggests that a Na^+, K^+ATPase that is found in virtually all eucaryotic cells is present also in the cancer cell lines PC3, P31 and U251 MG. The Na^+, K^+ATPase is not identical in all eukaryotic cells, and it has been observed that the catalytic and ouabain-binding subunit (α subunit) exists in at least three different forms (α 1, 2 and 3) [18]. The high sensitivity of PC3, P31 and U251 MG to ouabain suggests that their Na^+, K^+ATPase does not contain the α 1 subunit that exists, for example, in the rat kidney, but is rather equipped with the α 2 or α 3 subunit [18]. In the malignant glioma U251 MG, ouabain (0.01–1 mM) inhibited approximately 80% of the 15-min $^{86}Rb^+$ influx whereas in the prostatic (PC3) and pulmonary cancer (P31) cell lines, the inhibition by ouabain amounted to 65–70%. These values are rather high when compared to those in HT 29 human colonic adenocarcinoma cells [19], L1210 murine leukaemia cells [9] and rat astrocytes in primary culture [20], which suggests that the Na^+, K^+ATPase activity in U251 MG, PC3 and P31 is rather high. It can be assumed that this high activity is of importance

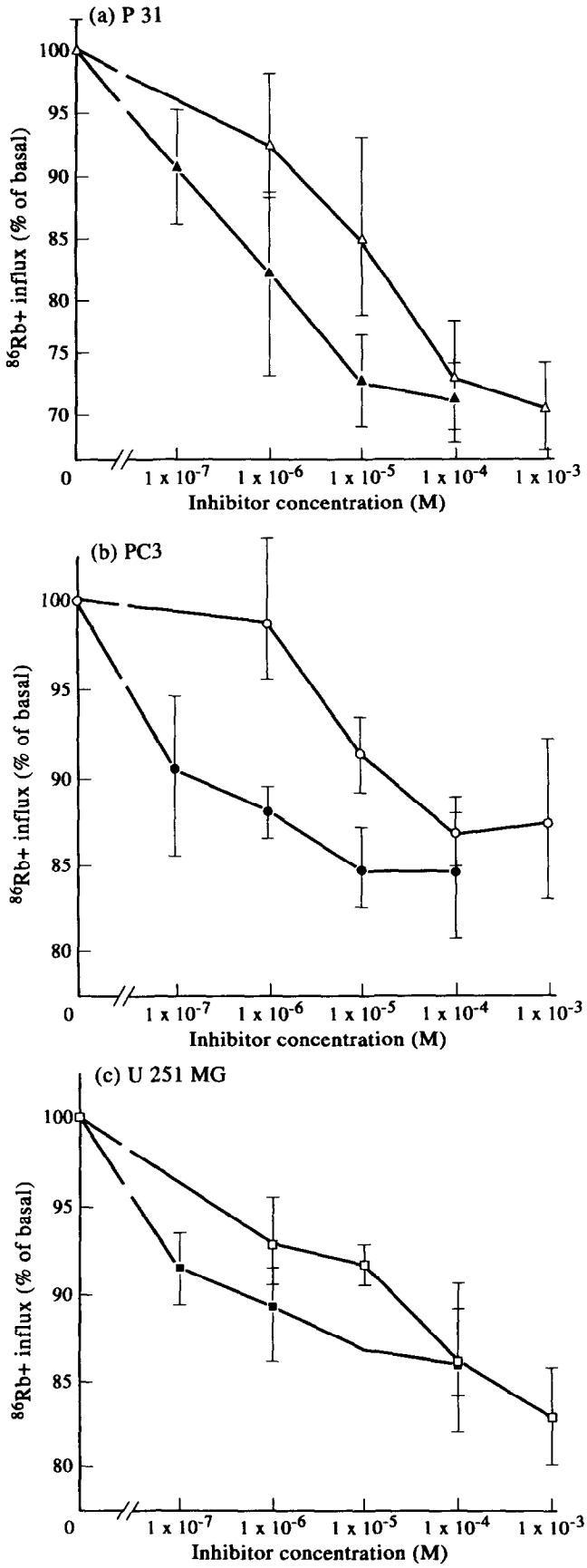

Figure 3. Effect of bumetanide and furosemide on $^{86}Rb^+$ influx in (a) P31; (b) PC3; (c) U251 MG. After 30 min incubation in Eagle's MEM with fetal calf serum and with or without bumetanide or furosemide, the cells were incubated for 15 min with the same medium supplemented with 28 μm ^{86}RbCl. Data are expressed as mean values ± S.E.M. (n = 6). Closed symbols refer to bumetanide, open symbols refer to furosemide.

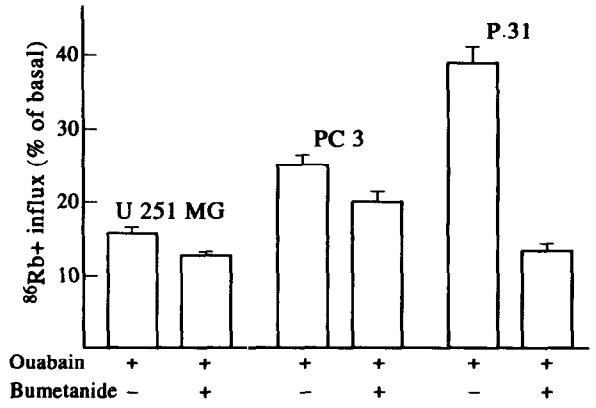

Figure 4. Interaction between ouabain and bumetanide on $^{86}Rb^+$ influx in PC3, P31 and U251 MG. After 30 min incubation in Eagle's MEM with 10% fetal calf serum and 100 uM ouabain and with or without 100 uM bumetanide, the cells were incubated for 15 min with the same medium supplemented with 28 μM ^{86}RbCl. Data are expressed as mean values ± S.E.M. (n = 6).

Table 1. Effect of tetraethylammonium on $^{86}Rb^+$ influx in the cell lines PC3, P31 and U251 MG

Modification of medium	$^{86}Rb^+$ influx (% of control = 100%)		
Tetraethylammonium (mM)	U251 MG	PC3	P31
0.01	94.5 ± 3.3	102.7 ± 3.7	106.3 ± 4.5
0.1	88.3 ± 2.7	91.3 ± 1.6	90.4 ± 2.8
1	89.2 ± 5.0	92.0 ± 2.2	84.1 ± 3.9
10	90.9 ± 6.1	88.5 ± 1.8	84.4 ± 3.7

After 30 min incubation in Eagle's minimal essential medium with 10% fetal calf serum and with or without test substances, the cells were incubated for 15 min in the same medium supplemented with 28 μM ^{86}RbCl. Data are expressed as mean values ± S.E.M. for seven to eight different experiments.

for the rapid growth of the cells since mitogenic stimulation of cell growth is associated with increased Na^+, K^+ATPase activity [5, 21]. Furthermore, the idea that ouabain-sensitive cation transport is important for cell growth was strengthened by our finding that ouabain (100 μM) strongly reduced the growth of all three cell lines.

The 5-sulphamoylbenzoic compounds, furosemide and bumetanide, are clinically used as potent diuretic drugs, but they can also be used as tools to detect a co-transport system for chloride and cations in the plasma membrane [15–17]. In the present study, furosemide and even more markedly, bumetanide decreased the $^{86}Rb^+$ influx, indicating the existence of Na^+, K^+, Cl^- co-transport in the cell lines investigated. The sensitivity of $^{86}Rb^+$ fluxes in these cell lines to bumetanide and furosemide is very similar to that observed in other cell types [19, 20, 22], and the higher sensitivity to bumetanide is in good agreement with the fact that bumetanide is 10–100 times more potent than furosemide as an inhibitor of Na^+, K^+, Cl^- co-transport [17]. It has been argued that furosemide-sensitive $^{86}Rb^+$ fluxes are not indicative of Na^+, K^+, Cl^- co-transport since it is believed that furosemide is also an inhibitor of other transport systems [17]. Moreover, bumetanide and furosemide seem to be capable of reversible binding to the ATP binding site of the Na^+, K^+ATPase [23] and, therefore, it is a possibility that the effect of these

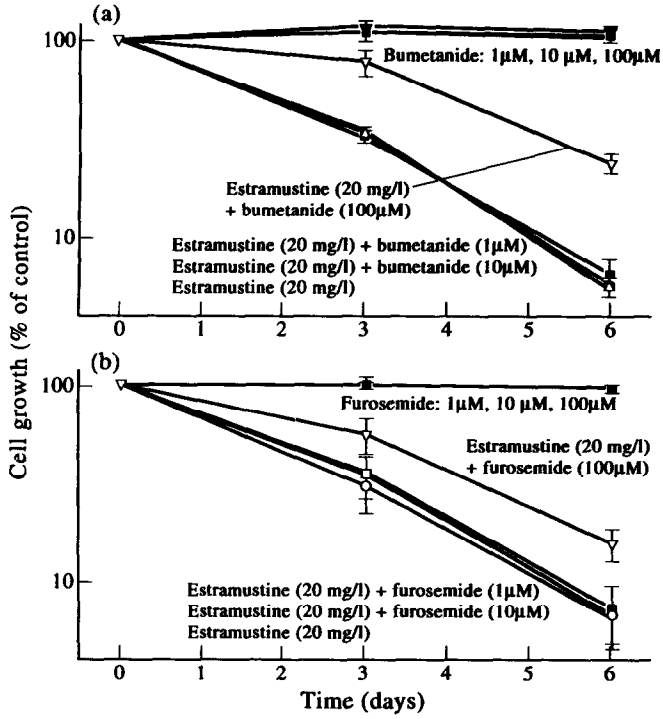

Figure 5. Effects of (a) bumetanide and (b) furosemide on the cytotoxicity of estramustine in the lung carcinoma cell line P31. Cells were incubated with estramustine ± test drugs for 6 days. The growth is expressed as % of control, not treated with test drugs, for nine separate experiments.

drugs on $^{86}Rb^+$ influx in P31 and PC3 is mediated by inhibition of this specific enzyme. However, bumetanide is considered a more specific inhibitor of this system [15], and our present observation, that furosemide and bumetanide inhibit $^{86}Rb^+$ influx in P31, PC3 and U251 MG to exactly the same extent, strongly suggests that the cells are equipped with a Na^+, K^+, Cl^- co-transport system. Moreover, the observation that 100 μM bumetanide also inhibited $^{86}Rb^+$ influx in the presence of the Na^+, K^+ATPase inhibitor ouabain indicates that the effect of loop diuretics on $^{86}Rb^+$ influx in P31, PC3 and U251 MG is mediated by an effect on a Na^+, K^+, Cl^- co-transport system rather than being secondary to inhibition of the Na^+, K^+ATPase. It could be argued that the concentrations of bumetanide and furosemide used are relatively high, and thus of no direct clinical relevance. However, according to Dollery's therapeutic drugs [24], an intravenous dose of 40 mg should give a furosemide concentration peak of 6 mg/l which corresponds to approximately 18 μmol/l. Given higher doses, serum concentrations of up to 30 mg/l (91 μmol/l) are well tolerated without signs of toxicity. In our *in vitro* experimental situation, considerable inhibition of ^{86}Rb influx was seen in most cases at 1 μmol/l and was very pronounced at 10 μmol/l for all three lines (Figure 3). For the bulk of our experiments, we used 100 μmol/l just to assure maximal inhibition of these potassium flux pathways.

The loop diuretic-sensitive Na^+, K^+, Cl^- co-transport seems to play an important role in cell proliferation. Thus, Swiss 3T3 cells lacking this transport system showed decreased proliferative response to phorbol esters [25], and mitogenic stimulation of cultured fibroblasts was associated with increased Na^+, K^+, Cl^- co-transport activity [3]. In a recent study, it was shown that the alkylating agent, nitrogen mustard, selectively inhibits loop diuretic-sensitive Na^+, K^+, Cl^- co-transport in L1210 leukaemia cells [9]. The detection of a Na^+, K^+, Cl^- co-transport

system in P31, PC3 and U251 MG may, therefore, prove to be important in forthcoming studies on cell growth and the action of anti-neoplastic drugs in these cells. The effects of bumetanide and furosemide with regard to the reduced estramustine cytotoxicity in solely lung carcinoma (P31) cells cannot, at present, be fully explained, and must be further evaluated. Currently, there is no direct or indirect evidence for interaction between the diuretics and the cytostatics. When measured in a spectophotometer the absorbance curves of a mixture of the drugs was similar to the absorbance values obtained when the values of single drug absorbances were added together (not shown). In this respect, it is of interest to recall earlier results which show that estamustine in a dose-dependent manner inhibited the $^{86}Rb^+$ fluxes in transformed fibroblasts and glioma cells [8]. Thus, in addition to the microtubuli system [8], the level of plasma membrane seems to be of interest when evaluating the mechanisms of action of estramustine cytotoxicity.

In summary, the present data suggest that at least three different K^+ flux pathways exist in U251 MG, PC3 and P31, i.e. Na^+, K^+ATPase, Na^+, K^+, Cl^- co-transport system and K^+ channels with high conductance. The identification of Na^+, K^+ATPase and Na^+, K^+, Cl^- co-transport system might be of particular interest since they play key roles in cell proliferation [3, 5], and might be primary targets for anti-neoplastic drugs [7, 9]. The reduced cytotoxicity by estramustine in P31 cells following incubation with furosemide and bumetanide strengthens this assumption. Further studies evaluating the role of these cation pathways in terms of targets for cancer treatment and in the sense of interaction in the clinical situation between such pharmaceuticals and the direct cancer treatment are certainly justified.

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Acknowledgements—This study was supported by grants from the Swedish Cancer Society, Lion's Cancer Research Foundation, Umeå, Sweden and the Swedish Medicaal Research Council.