3. Erlichman C, Fine S, Wong A, Elhakim T. A randomized trial of fluorouracil and folinic acid in patients with metastatic colorectal carcinoma. *J Clin Oncol* 1988, **6**, 469–475.
4. Dreyfuss AI, Clark JR, Wright JE, *et al.* Continuous infusion of high-dose leucovorin with 5-fluorouracil and cisplatin for untreated stage IV carcinoma of the head and neck. *Ann Int Med* 1990, **11**, 167–172.
5. Swain SM, Lippman ME, Egan EF, Drake JC, Steinberg SM, Allegra CJ. Fluorouracil and high-dose leucovorin in previously treated patients with metastatic breast cancer. *J Clin Oncol* 1989, **7**, 890–899.
6. Vokes EE, Vijayakumar S, Hoffman PC, *et al.* 5-fluorouracil with oral leucovorin and hydroxyurea and concomitant radiotherapy for stage III non-small cell lung cancer. *Cancer* 1990, **66**, 437–442.
7. Milano G, Etienne MC, Thyss A, *et al.* Evidence for CSF accumulation of 5-methyltetrahydrofolate during repeated courses of methotrexate plus folinic acid rescue. *Br J Cancer* 1989, **59**, 627–630.
8. Christophidis N, Mihaly G, Vajda F, Louis W. Comparison of liquid-and gas-liquid chromatographic assays of 5-fluorouracil in plasma. *Clin Chem* 1979, **25**, 83–86.
9. Pinguet F, Favre G, Canal P, Pujol A, Soula G. Stability of 5-fluorouracil to heat and light. *J Pharm Clin* 1990, **9**, 155–158.
10. Lecompte D,Bousselet M, Gayrard D, Poitou P. Stability study of reconstituted and diluted solutions of calcium folinate. *Pharm Ind* 1991, **53**, 90–94.
11. Smith JA, Morris A, Duafala ME, Bertino JR, Markman M, Kleinberg M. Stability of floxuridine and leucovorin calcium admixtures for intraperitoneal administration. *Am J Hosp Pharm* 1989, **46**, 985–989.
12. Piedbois P, Gimonet JF, Feuilhade F, *et al.* 5-FU, folinic acid and alpha-2a- interferon combination in advanced gastrointestinal cancer. *Proc ASCO* 1991, **10**, 141.
13. Anderson N, Lokich J, Bern M, Wallach S, Moore C, Williams D. A phase I clinical trial of combined fluoropyrimidines with leucovorin in a 14-day infusion. Demonstration of biochemical modulation. *Cancer* 1989, **63**, 233–237.

Eur J Cancer, Vol. 29A, No. 1, pp. 132–137, 1993.
Printed in Great Britain

0964–1947/93 $5.00 + 0.00

Tumour Cell Proliferation is Abolished by Inhibitors of Na^+/H^+ and HCO_3^-/Cl^- Exchange

Branka Horvat, Shahram Taheri and Aida Salihagić

Cell membrane-associated ion transporters, Na^+/H^+ exchanger and Na^+-dependent HCO_3^-/Cl^- antiport, were shown to be important in the regulation of acidic intracellular pH in different cell types. This study investigated the role of the ion exchangers and their inhibitors in the serum-induced proliferation of two murine tumour cell lines, P815 and L929. The presence of Na^+/H^+ exchanger [inhibited by amiloride and 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA)] and Na^+-dependent HCO_3^-/Cl^- antiport [inhibited by 4,4′diisothiocyanostilben-2,2-disulphonic acid (DIDS)] was shown on the tumour cell line tested. EIPA suppressed tumour cell proliferation more strongly than amiloride, and its effect was further increased after intracellular acidification by nigericin. DIDS slightly inhibited proliferation of L929 cell line and did not influence proliferation of P815 cells. However, in nigericin acidified cells DIDS had a dose dependent antiproliferative effect. Furthermore, DIDS significantly increased antiproliferative effects of amiloride and EIPA, suggesting the activity of Na^+-dependent HCO_3^-/Cl^- antiport in tumour cell proliferation. These results demonstrate the importance of Na^+-dependent HCO_3^-/Cl^- exchange in addition to Na^+/H^+ antiport, in tumour cell proliferation and indicate the possibility that ion exchange inhibitors could act as antitumour reagents.
Eur J Cancer, Vol. 29A, No. 1, pp. 132–137, 1993.

INTRODUCTION

THE MICROENVIRONMENT in tumours is generally more acidic than in normal tissues, approximately 0.5 pH units below the pH of normal tissues. The reduction of tumour pH is caused by the increased production of lactic acid and hydrolysis of ATP under hypoxic conditions [1, 2]. Maintaining the viability of tumour cells under such conditions requires regulation of intracellular pH (pHi) [2, 3]. Major transport mechanisms in regulation of decreased pHi involve Na^+/H^+ antiport and sodium-dependent HCO_3^-/Cl^- exchanger [4, 5].

Na^+/H^+ exchange seems to be important in initiation of growth and proliferation of different cell types, in addition to its role in regulation of cytoplasmic concentrations of Na^+, H^+ and cell volume [6–8]. The activation of Na^+/H^+ exchange by various mitogens and growth factors leads to the intracellular alkalinisation followed by cell growth and proliferation [6, 7]. Recent investigations have shown that mutant cells which lack Na^+/H^+ exchange have either absent or reduced ability to generate tumours [9, 10]. Although Na^+/H^+ antiport seems to be the major cellular H^+-extruding mechanism, sodium-dependent HCO_3^-/Cl^- antiport has been detected in a number of cell lines [11]. This exchanger was shown to be important in the regulation of small reduction of pHi and recovery of steady-state pHi [12]. However, mechanism of activation of Na^+-dependent HCO_3^-/Cl^- antiport is still poorly understood and its role in cell proliferation has not been previously shown.

We have demonstrated the presence of Na^+/H^+ antiport and Na^+-dependent HCO_3^-/Cl^- exchanger in a tumour cell line and

Correspondence to B. Horvat, Centre d'immunologie de Marseille-Luminy Parc Scientifique de Luminy-Case 906, 13288 Marseille Cedex 9, France; B. Horvat, S. Taheri and A. Salihagić are at the Department of Physiology, School of Medicine, Zagreb University, Croatia.
Revised and accepted 8 May 1992.

showed that inhibitors of both ion exchangers prevent an increase of intracellular pH in the presence of the Na^+-containing medium. In addition, we found that serum induced proliferation of two tumour cell lines could be markedly suppressed with inhibitors of Na^+/H^+ and HCO_3^-/Cl^- antiports, indicating the potential role of both antiport systems in cell proliferation.

MATERIALS AND METHODS

Cell lines

Murine mastocitoma cell line, P815 (ATCC TIB 64) and murine fibrosarcoma L929 (obtained from Dr Z. Dembic, Hoffmann-La Roche, Basel, Switzerland) were maintained in modified Eagle's medium (MEM) (Gibco Laboratories), supplemented with 10% fetal calf serum (FCS), 50 U/ml neomycin, 50 U/ml polymixin, 5×10^{-5} mol/l 2-mercaptoethanol, 2.2 g/l sodium bicarbonate and 15 mmol/l Hepes, at 37°C in an incubator with a humidified atmosphere of 5% CO2. Proliferation experiments were carried out with cells arrested for 24 h in MEM medium, where FCS was replaced with 0.1% bovine serum albumin (BSA).

Reagents

Nigericin, 4,4'diisothiocyanostilben-2,2'-disulphonic acid (DIDS), trypsin and bovine serum albumin (BSA) were purchased from the Sigma Chemical Co. Amiloride was obtained from Merck, Sharp and Dohme, Res. Lab. and 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA) had been synthesised as previously described [13]. Acetoxymethyl ester of BCECF [2'7'-*bis* (2-carboxyethyl)-5 (and 6)-carboxyfluorescein] was purchased from Molecular Probes, and acetozalamide sodium from Lederle Parenterals Inc. Stock solutions of DIDS, EIPA and BCECF were made in dimethylsulphoxide (DMSO), purchased from Aldrich Chemical Comp. Final concentration of DMSO in solutions of DIDS and EIPA did not exceed 0.1%, which was shown in control experiments not to interfere with cell proliferation.

Fluorescence measurement of intracellular pH

Intracellular pH was measured by the pH-sensitive, fluorescent dye BCECF, as described previously [14–16]. The method is based on the penetration of the acetoxy-methyl ester of BCECF into cells, where it is cleaved by internal esterases, releasing the poorly permeant and highly fluorescent BCECF. There is a linear relationship between its fluorescence intensity and pHi within the range of 6.5 and 7.5. 3×10^6 cells were loaded with acetoxymethyl ester of BCECF (4 μg/ml) for 60 min at room temperature, in serum free MEM, sedimented afterwards and resuspended in Na^+, and HCO_3^- free buffer (140 mmol/l TMACl, 10 mmol/l glucose, 1 mmol/l KCl, 1 mmol/l $CaCl_2$, 1 mmol/l $MgCl_2$/Hepes–Tris, pH = 7.1). Measurements of pHi were carried out in a Ratio Farrand Optical Co. Inc. spectrofluorimeter (excitation filter 490 nm; emission filter 511 nm). Cells (10^6) were placed into a cuvette and acidified by K^+/H^+ ionophore nigericin (4 μg/ml) and appearance of an amiloride-sensitive alkalinisation, as an indicative of Na^+/H^+ exchange, was recorded following addition of 100 mmol/l NaCl.

Activity of the Na^+-dependent HCO_3^-/Cl^- antiport was demonstrated in similar experiments (17). Cells (2×10^6) were added to the Na^+, and HCO_3^- free buffer and acidified with nigericin. After inhibition of Na^+/H^+ exchanger with amiloride, $KHCO_3$ (25 mmol/l) was added and the increase of pHi, which could be reproducibly detected, reflected the activity of Na^+-dependent HCO_3^-/Cl^- exchanger. Calibration of pHi versus fluorescence intensity was carried out in K^+-containing solution (140 mmol/l KCl, 10 mmol/l glucose, 1 mmol/l $CaCl_2$, 1 mmol/l $MgCl_2$/Hepes–Tris, pH = 6.5–7.5) with nigericin (4 μg/ml), as described previously [18].

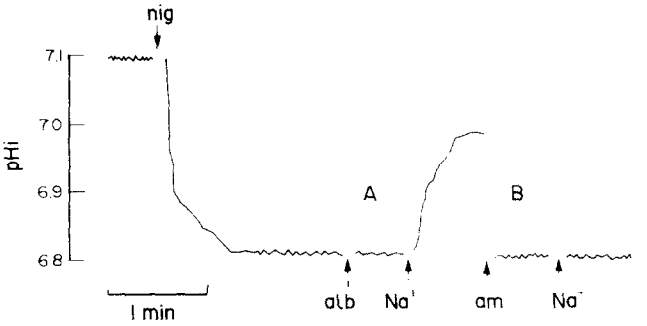

Fig. 1. Assessment of the presence of the Na^+/H^+ exchanger in P815 cells. Cells were loaded with BCECF and suspended in Na^+ and HCO_3^- free buffer (see Material and methods). Nigericin (nig, 4 μg/ml) was added to acidify the cytoplasm and albumin (alb, 2.5 mg/ml) was further added to bind excess nigericin. NaCl (Na^+, 100 mmol/l) allows exchange of extracellular Na^+ for intracellular H^+, leading to a rise in pHi (A), which is blocked by the addition of amiloride (am, 0.1 mmol/l) (B).

Cell proliferation measurement

Prior to the measurement of cell proliferation confluent cells were preincubated for 24 h in the MEM medium, where FCS was replaced with 0.1% BSA. To determine proliferation, cells were resuspended in medium with 10% FCS (regular medium) and placed in microtitre 96 well plates (Nunc), where 10^4 cells per well (P815) or 2×10^4 cells per well (L929) were incubated in the final volume of 200 μl. In addition, L929 cells were preincubated in regular medium for the further 2 h to obtain adherence. Reagents were diluted in the regular medium and added to cell cultures in the volume of 100 μl, while control cell cultures received 100 μl of the regular medium. Each culture received 3.7 mBq of [^{3}H]thymidine (925 GBq/mmol, Amersham International). After 18 h of incubation, at 37°C, in 5% CO_2 incubator, cultures were harvested with automatic cell harvester (Multimash 2000, Dunatech Lab. Inc. Sussex, UK) and [^{3}H]TdR uptake was determined in scintillation counter Beckman LS 1701.

Significant differences between two means were determined by the Student's *t*-test.

RESULTS

Presence of Na^+H^+ *antiport and* Na^+*-dependent* HCO_3^-/Cl *exchanger activity*

Fluorescence dye BCECF was used to investigate the presence of Na^+/H^+ antiport and Na^+-dependent HCO_3^-/Cl^- exchanger in P815 cells. Fig. 1(a) shows representative experiment where Na^+/H^+ exchanger was demonstrated by Na^+-induced alkalinisation of acid loaded cells. Cell acidification was achieved by the addition of K^+/H^+ ionophore, nigericin, to the cells resuspended in the medium with low K^+ concentration. The exchanger could be inhibited by 0.1 mmol/l amiloride (Fig. 1b). Identical inhibition was obtained with amiloride analog EIPA (10 μmol/l) (data not shown). In similar experiments activity of Na^+-dependent HCO_3^-/Cl^- exchanger was demonstrated in P815 cells (Fig. 2). Following intracellular acidification recovery of pHi was obtained after addition of HCO_3^- into buffer containing Na^+ and amiloride. Activity of Na^+/H^+ antiport was blocked

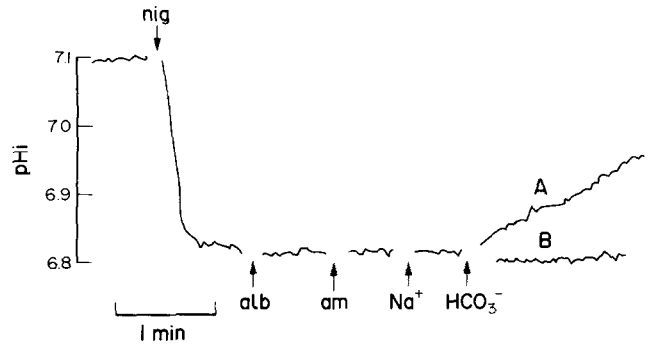

Fig. 2. Assessment of the presence of the Na^+-dependent HCO_3^-/Cl^- exchanger in P815 cells. Cells were loaded with BCECF and suspended in Na^+ and HCO_3^--free buffer (see Materials and methods). Nigericin (nig, 4 μg/ml) and albumin (alb, 2.5 mg/ml) were added at the times indicated. Na^+/H^+ exchange was blocked by amiloride (am, 0.1 mmol/l), buffer was supplemented with Na^+ (100 mmol/l), and pHi monitored after addition of 25 mmol/l $KHCO_3$ (A). The rise of pHi could be blocked if cells were preincubated with 0.1 mmol/l DIDS, for 15 min, at room temperature and resuspended into original buffer (B). Results are from the representative experiments.

in the presence of amiloride and HCO_3^--induced alkalinisation of P815 cells indicated the activity of Na^+-dependent HCO_3^-/Cl^- antiport (Fig. 2a). This activity could not be detected if Na^+ is absent in the buffer (data not shown) and could be inhibited after preincubation of cells with 0.1 mmol/l DIDS (Fig. 2b). These results were confirmed using ^{36}Cl efflux method (Salihagić *et al.*, in preparation). In L929 cells activity of Na^+/H^+ exchanger (inhibited by amiloride) as well as Na^+-dependent HCO_3^-/Cl^- exchanger (inhibited by DIDS) has been shown previously [11].

Effects of amiloride and DIDS on cell proliferation

We analysed the role of Na^+/H^+ and HCO_3^-/Cl^- exchangers in tumour cell proliferation. Before each experiment cells were incubated in medium where FCS was replaced with 0.1% BSA, to obtain growth arrest. Tumour cell proliferation was stimulated with 10% FCS, added with the medium. We investigated whether specific inhibitors of Na^+/H^+ and HCO_3^-/Cl^- exchange proteins, amiloride and DIDS, respectively, could alter proliferation of L929 cells. Figure 3 shows that both inhibitors could suppress cell proliferation, although marked suppressive effect is seen only at the concentration of 10^{-3} mmol/l, a concentration that was shown to be cytotoxic for cells, as described later. However, when present together in culture, amiloride and DIDS significantly inhibited cell proliferation, much more strongly than either reagent tested alone ($P < 0.01$, t-test). Similar results were obtained with cells taken from the culture in the exponential phase of growth, but [^{3}H]thymidine incorporation in these cultures was lower (data not shown).

DIDS is known to inhibit all bicarbonate-dependent exchangers in the cell membranes [5]. The ability of DIDS to increase antiproliferative effects of amiloride indicated that it is most likely acting on a Na^+-dependent HCO_3^-/Cl^- exchanger, preventing intracellular alkalinisation. We confirmed these data by analysing the role of the other DIDS sensitive HCO_3^--transporters in tumour cell proliferation. Na^+-independent HCO_3^-/Cl^- exchange and Na^+-HCO_3^- cotransport require intracellular generation of H_2CO_3, catalysed by carbonic anhydrase [4, 19]. We tested whether a specific inhibitor of carbonic anhydrase, acetozalamide, could interfere with cell proliferation in the concentration previously shown to block Na^+-HCO_3^- cotransport [20]. However, acetozalamide did not modulate proliferation of either L929 or P815 cells even when higher concentration of the drug was used (data not shown), indicating that intracellular generation of H_2CO_3 is not necessary for proliferation of tested tumour cells. In addition, we were not able to demonstrate the presence of DIDC-sensitive cellular acidification in tested cells (data not shown).

All reagents, which were examined for their effects on cell proliferation, were tested for potential cytotoxic effects on L929 and P815 cells, using spectrophotometric assay [21] and ^{51}Cr-release assay [22], respectively (data not shown). Except for the DIDS and amiloride in concentration 10^{-3} mol/l, none of the reagents, in the concentration used in the proliferation assay, influenced cell viability, indicating that their antiproliferative effects were not result of nonspecific cytotoxic activity. In addition, it has been reported previously that neither amiloride (10^{-4} mol/l) nor DIDS (10^{-4} mol/l) influence plating efficiency when applied either alone or in combination with nigericin in cell culture exposed to the initial extracellular pH 7–7.2 [3].

Nigericin potentiates effects of ion exchange inhibitors

Nigericin is an ionophore which causes intracellular acidification by allowing entry of protons in exchange of K^+, which leaves the cell down its chemical gradient [18]. We have tested whether intracellular acidification could influence tumour cell proliferation. Figures 1 and 2 show that nigericin caused immediate decrease of intracellular pH in P815 cells, loaded with BCECF. Addition of nigericin to either P815 or L929 cell culture, stimulated with 10% FCS, caused significant dose dependent inhibition of tumour cell proliferation (Fig. 4a). In further experiments, nigericin was used in the combination with the other ion exchange inhibitors, in the concentration in which it induced 50% reduction of the proliferation (0.25 μg/ml).

Role of Na^+/H^+ exchange in cell proliferation was further analysed using highly potent synthetic amiloride analog EIPA, shown to be as much as 200-fold more effective than amiloride [23]. EIPA was added in various concentrations to tumour cell cultures and cell proliferation was determined in six individual experiments. In all assays EIPA caused significant dose dependent inhibition of tumour cell proliferation of either L929 cells

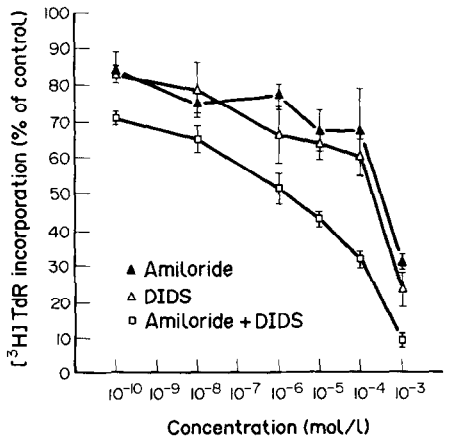

Fig. 3. Effects of amiloride, DIDS and their combination on the proliferation of L929 cells. Cells were incubated for 18 h with indicated concentrations of drugs, precipitated afterwards, and [^{3}H]thymidine (TdR) incorporation was determined by scintillation counting. Results are expressed as percentage of ^{3}H-TdR incorporation in the absence of drug [mean (S.E.)], from five separate experiments, each done in triplicate.

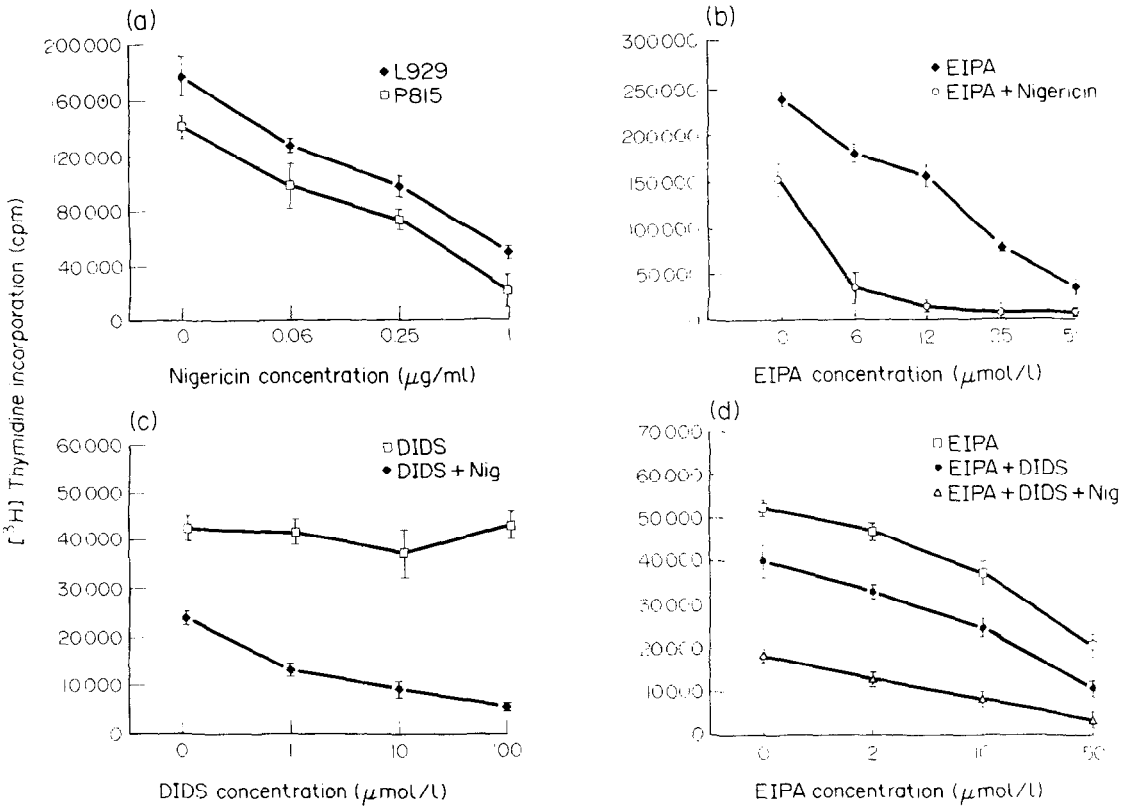

Fig. 4. Proliferation of P815 (a, c and d) and L929 (a and b) cells exposed to the different concentrations of nigericin, EIPA, DIDS or their combinations. In the experiments shown in b, c, and d nigericin was used in the concentration 0.25 μg/ml. Cells were incubated for 18 h with indicated concentrations of drugs, precipitated afterwards, and [³H] thymidine (TdR) incorporation was determined by scintillation counting. (d) DIDS was added to the culture in the concentration 100 μmol/l; nigericin added alone to the culture, reduced proliferation to 33 283 (2213) cpm. Data are presented as a mean of triplicate cultures (S.E.), from a representative experiment.

(Fig. 4b), or P815 cells (Fig. 4d). Moreover, when cells were acidified with nigericin, the antiproliferative effects of EIPA were greatly enhanced in L929 cells (Fig. 4b).

Addition of inhibitor of HCO_3^-/Cl^- exchanger, DIDS, to P815 cell culture did not modulate cell proliferation (Fig. 4c), indicating that DIDS, in the concentrations between 10^{-7} and 10^{-4} mol/l does not interfere with proliferation of tested cells. However, acidification of P815 cells by nigericin allowed DIDS to express significant dose dependent inhibition of cell proliferation. In the concentrations ranging from 10^{-4} to 10^{-6} mol/l DIDS had significantly higher inhibitory effect if combined with nigericin, compared to the nigericin alone ($P < 0.01$, t-test), suggesting the activity of Na^+-dependent HCO_3^-/Cl^- exchanger in the proliferation of acidified cells.

These observations were further supported by results showed on Fig. 4d (one of three similar experiments). Although DIDS did not influence proliferation of P815 cells by itself, when added together with EIPA, it significantly increased the antiproliferative effects of EIPA ($P < 0.01$, t-test) (Fig. 4d). Additional acidification of cells with nigericin, allowed EIPA and DIDS to exert the highest inhibitory effect on proliferation of P815 cells, significantly different from other combinations tested ($P < 0.01$, t-test) (Fig. 4d).

DISCUSSION

Regulation of intracellular pH is essential for the normal function of cells, which contain a number of strongly pH-dependent enzymes [4]. Principal membrane-based ion transport mechanisms known to contribute to the regulation of decreased pHi in many cell types include Na^+/H^+ antiport and Na^+-dependent HCO_3^-/Cl^- exchanger [2, 5]. Reduced pH often found in tumours could additionally stimulate activity of these exchangers [2]. This study investigated the role of Na^+/H^+ antiport and Na^+-dependent HCO_3^-/Cl^- exchanger in the serum stimulated proliferation of two tumour cell lines, induced after serum deprivation.

We identified the activity of Na^+/H^+ antiporter in tumour cell line P815. This antiport could be blocked by either amiloride, or its synthetic analog EIPA. We also showed the presence of a Na^+-dependent HCO_3^-/Cl^- exchanger in P815 cells, which could be blocked by DIDC. The activity of Na^+/H^+ exchanger, as well as Na^+-dependent HCO_3^-/Cl^- exchanger in L929 cells has been shown previously [11].

We had further analysed the role of the Na^+/H^+ exchanger in tumour cell proliferation induced by growth factors present in fetal calf serum. Ability of serum to stimulate amiloride-sensitive Na^+/H^+ exchange has been shown before [5, 24]. Most of the previous studies, showing importance of Na^+/H^+ antiport have used a HCO_3^--free system [25] which does not reflect the physiological conditions. All our proliferation experiments were done in medium which contained bicarbonates, and cells were cultured in the incubator with 5% CO_2. When amiloride was added to tumour cell cultures, a slight decrease in tumour cell proliferation was detected, mainly with higher concentrations of the drug. As amiloride was shown in high concentrations (over 100 μmol/l) to introduce side effects, such as inhibition of protein synthesis [25, 26], we have tested another specific inhibitor of Na^+/H^+ exchanger, EIPA. In all our experiments

EIPA showed marked inhibition of tumour cell proliferation. Equivalent inhibitory effects of EIPA were obtained in granulocyte–macrophage colony-stimulating factor induced proliferation of leukaemic cells [27], although amiloride analogues were inactive in interleukin 2 induced lymphocyte proliferation [28]. However, we did not obtain complete inhibition of cell proliferation by EIPA, indicating the presence of the other pH regulating mechanisms, important for the cell proliferation in the environment where HCO_3^- ions are present.

We continued the study analysing the role of HCO_3^-/Cl^- exchange in tumour cell proliferation using stilben derivate DIDS. Although DIDS inhibited both Na^+ dependent and Na^+ independent HCO_3^-/Cl^- antiport, we could expect activity of Na^+ dependent exchanger, mostly stimulated in proliferating tumour cells by acid load, caused by accumulation of metabolically generated acids (2). Na^+ independent HCO_3^-/Cl^- exchanger acts as a cell acidifying mechanism and promotes pHi recovery, following an alkaline load [5]. Consequently, this antiport antagonises activity of both Na^+/H^+ and Na^+-dependent HCO_3^-/Cl^- exchanger. The importance of other mechanisms of HCO_3^--transport across the membrane in cell proliferation was further excluded, demonstrating that a specific inhibitor of carbonic anhydrase does not modulate proliferation. Addition of DIDS into tumour cell culture caused slight decrease in tumour cell proliferation in L929 cells, and had no effect on P815 cells. However, when P815 cells were further acidified by the addition of nigericin, DIDS expressed dose dependent antiproliferative effect. Presence and the activity of Na^+ dependent HCO_3^-/Cl^- exchanger was shown to be quite variable among different cells lines [11], which could explain different sensitivity to DIDS in our two tested lines. Besides its role in regulation of pHi, Na^+-dependent HCO_3^-/Cl^- exchanger was found to be important in cell function of proximal convoluted tubules of the kidney [29, 30], but the role of this antiport in the cell proliferation, has to our knowledge not been reported.

When inhibitors of Na^+/H^+ antiport and HCO_3^-/Cl^- exchanger are present together in tumour cell culture, DIDS significantly potentiated antiproliferative effects of either amiloride or EIPA. Although DIDS alone did not influence proliferation of P815 cells at all, it significantly increased effects of EIPA, indicating the activity of Na^+ dependent HCO_3^-/Cl^- exchanger in this cell line. These results suggest that Na^+/H^+ antiport could take over the role of blocked Na^+-dependent HCO_3^-/Cl^- exchanger, but the opposite is not the case. Role of Na^+-dependent HCO_3^-/Cl^- exchanger appeared to be critical for cell proliferation only when Na^+/H^+ antiport was blocked, and inhibition of both ion transporters markedly abolished tumour cell proliferation.

We further analysed the role of membrane ion transporters, using nigericin, the ionophore which lowers pHi by allowing exchange of intracellular K^+ for extracellular H^+ [18]. As many solid tumours are usually faced with higher acidic load [2], an increase activity of H^+ extrusion mechanisms could be expected. Nigericin had dose dependent suppressive activity on [^{3}H] thymidine incorporation in tested cells, indicating that intracellular acidification could significantly decrease tumour cell proliferation. In addition, nigericin significantly potentiated antiproliferative effects of EIPA and DIDS. In accordance with these results it was shown previously that in conditions with low extracellular pH, nigericin, amiloride and DIDS were cytotoxic for tumour cells, representing a potential new class of anticancer drugs [3]. Ion exchange inhibitors may have effects on the normal as well as on tumour cells. However, these effects could be further augmented in tumours due to the conditions present in tumour tissue (acidity and hypoxia, [1, 2]). In addition, amiloride was previously shown to inhibit tumour growth *in vivo* [31]. Experiments designated to examine whether ion exchange inhibitors, tested in this study, could effect tumour cells *in vivo* are currently in progress.

In summary, this study showed that serum induced tumour cell proliferation is associated with the function of Na^+/H^+ and Na^+-dependent HCO_3^-/Cl^- ion transporters. Selective inhibitors of these antiport systems significantly decreased tumour cell proliferation. Further understanding of intracellular events involved in tumour cell growth could help in development of new anticancer reagents. Our results have shown that inhibitors of acid extrusion mechanisms in cell have stronger antiproliferative effect in reduced pHi, a condition often associated with tumours, which may enable them to selectively attack tumour cells *in vivo*.

1. Wike-Hodey JL, Haveman J, Reinhold JS. The relevance of tumor pH to the treatment of malignant disease. *Radiother Oncol* 1984, **2**, 343–366.
2. Tannock IF, Rotin D. Acid pH in tumors and its potential for therapeutic exploitation. *Cancer Res* 1989, **49**, 4373–4394.
3. Rotin D, Wan P, Grinstein S, Tannock I. Cytotoxicity of compounds that interfere with regulation of intracellular pH: a potential new class of anticancer drugs. *Cancer Res* 1987, **47**, 1497–1504.
4. Roos A, Baron WF. Intracellular pH. *Physiol Rev* 1981, **61**, 296–434.
5. Frelin C, Vigne P, Ladoux A, Lazdunski M. The regulation of the intracellular pH in cells from vertebrates. *Eur J Biochem* 1988, **174**, 3–14.
6. Grinstein S, Rothstein A. Mechanisms of regulation of the Na^+/H^+ exchanger. *J Mem Biol* 1986, **90**, 1–12.
7. Seifter JL, Aronson PS. Properties and physiological roles of the plasma membrane sodium–hydrogen exchanger. *J Clin Invest* 1986, **78**, 859–64.
8. Salihagić A, Macković, Banfić H, Sabolić I. Short-term and long-term stimulation of Na^+/H^+ exchange in cortical brush-border membranes during compensatory growth of the rat kidney. *Pflügers Arch* 1988, **413**, 190–196.
9. Lagarde AE, Franchi AJ, Paris S, Poussegur JM. Effect of mutations affecting Na^+/H^+ antiport activity on tumorigenic potential of hamster lung fibroblasts. *J Cell Biochem* 1988, **36**, 249–260.
10. Rotin D, Steele-Norwood D, Grinstein S, Tannock I. Requirement of the Na^+/H^+ exchanger for tumor growth. *Cancer Res* 1989, **49**, 205–211.
11. Reinertsen KV, Tonnassen TI, Jacobsen J, Sandvig K, Olsnes S. Role of chloride/bicarbonate antiport in the control of cytosolic pH. Cell-line differences in activity and regulation of antiport. *J Biol Chem* 1988, **263**, 11117–11125.
12. Tonnessen TI, Sandvig K, Olsnes AS. Role of Na^+/H^+ and Cl^-/HCO_3^- antiports in the regulation of cytosolic pH near neutrality. *Am J Physiol* 1990, **258**, C1117–C1126.
13. Cragoe EJ Jr, Woltersdorf OW, Bicking S, Kwong F, Jones JH. Pyrazine diuretics. II. *N*-amidino-5-substituted-6-halopyrazinecarboxamides. *J Med Chem* 1976, **10**, 66–77.
14. Grinstein S, Cohen S, Rothstein A. Cytoplasmic pH regulation in thymic lymphocytes by an amiloride-sensitive Na^+/H^+ antiport. *J Gen Physiol* 1984, **83**, 341–369.
15. Rink TJ, Tsien RY, Pozzam T. Cytoplasmic pH and free Mg^{2+} in lymphocytes. *J Cell Biol* 1982, **95**, 189–196.
16. Grinstein S, Clarke CA, Rothstein A. Activation of Na^+/H^+ exchange in lymphocytes by osmotically-induced volume changes and cytoplasmic acidification. *J Gen Physiol* 1983, **82**, 619–638.
17. Ladoux A, Krawice I, Cragoe EJ Jr, Abita JP, Frelin C. Properties of the Na^+-dependent HCO_3^-/Cl^- exchange system in U937 human leukemic cells. *Eur J Biochem* 1987, **170**, 43–49.
18. Thomas JA, Buchsbaum RN, Zimniak A, Racker E. Intracellular pH measurements in Erlich ascites tumor cells utilizing spectroscopic probes generated *in situ*. *Biochemistry* 1979, **18**, 2210–2216.
19. Kinckelbein R, Aronson PS, Schron CM, Seifter J, Dobbins JW. Sodium and chloride transport across rabbit ileal brush border. II

Evidence for Cl^--HCO_3^- exchange and mechanism of coupling. *Gastrointest Liver Physiol* 1985, **12**, G236–G245.
20. Soleimani A, Aronson SP. Effects of acetozalamide on Na^+-HCO_3^- cotransport in basolateral membrane vesicles isolated from rabbit renal cortex. *J Clin Invest* 1989, **83**, 945–931.
21. Haranaka K, Stomi N, Sakurai A. Differences in tumour necrosis factor productive ability among rodents. *Br J Cancer* 1984, **50**, 471–478.
22. Horvat B, Loukides JA, Anandan L, Brewer E, Flood PM. Production of interleukin 2 and interleukin 4 by immune $CD4^+CD8^+$ cells and their role in the generation of antigen-specific cytotoxic T cells. *Eur J Immunol* 1991, **21**, 1863–1871.
23. Vigne P, Frelin C, Cragoe EJ, Lazdunski M. Structure activity relationships of amiloride and certain its analogues in relation to the blockade of the Na^+/H^+ exchange system. *Mol Pharmacol* 1984, **25**, 131–136.
24. Moolenaar WH, Mummery CL, van der Saag PT, de Laat SW. Rapid ionic events and the initiation of growth in serum-stimulated neuroblastoma cells. *Cell* 1981, **23**, 789–798.
25. L'Allemain G, Franchi A, Cragoe E Jr, Pouyssegur J. Blockade of Na^+/H^+ antiport abolishes growth factor induced DNA synthesis in fibroblasts. Structure-activity relationship in the amiloride series. *J Biol Chem* 1984, **259**, 4313–4319.
26. Leffert HL, Koch KS, Fehlmann M, Heiser W, Lad PJ, Skelly H. Amiloride blocks cell-free protein synthesis at levels attained inside cultured rat hepatocytes. *Biochem Biophys Res Commun* 1982, **107**, 738–749.
27. Caracciolo D, Pannocchia A, Treves S, *et al.* Role of Na^+/H^+ exchange in the granulocyte–macrophage colony-stimulating factor-dependent growth of a leukemic cell line. *J Cell Physiol* 1990, **143**, 133–139.
28. Mills GB, Cragoe EJ, Gelfand EW, Grinstein S. Interleukin 2 induces a rapid increase in intracellular pH through activation of a Na^+/H^+ antiport. Cytoplasmic alkalinization is not required for lymphocyte proliferation. *J Biol Chem* 1985, **260**, 12500–12507.
29. Biagi BA, Sohtell M. Electrophysiology of basolateral bicarbonate transport in the rabbit proximal tubule. *Am J Physiol* 1986, **250**, F267–F272.
30. Sasaki S, Shiigai T, Yoshiyama N, Takeuchi J. Mechanism of bicarbonate exit across basolateral membrane of rabbit proximal straight tubule. *Am J Physiol* 1987, **252**, F11–F18.
31. Sparks RL, Pool TB, Smith NKR, Camron IL. Effects of amiloride on tumor growth and intracellular element content in tumor cells *in vivo*. *Cancer Res* 1983, **43**, 73–77.

Acknowledgements—We are grateful to Dr. Amela Hadžić, for the help during initiation of this work, and to Dr. Hrvoje Banfić and Dr. Filip Čulo for the critical review of the manuscript.

Eur J Cancer, Vol. 29A, No. 1, pp. 137–140, 1993.
Printed in Great Britain

0964–1947/93 $5.00 + 0.00

Anticlastogenic Effects of 13-*cis*-Retinoic Acid *in vitro*

Zoltán Trizna, T.C. Hsu, Stimson P. Schantz, J. Jack Lee and Waun K. Hong

The anticlastogenic effects of 13-*cis*-retinoic acid were studied in four human lymphoblastoid cell lines and in primary lymphocyte cultures derived from the peripheral blood of 11 study subjects. Cells were pre-incubated with 13-*cis*-retinoic acid in the concentration range of 10^{-8}–10^{-5} mol/l for 24 h and the numbers of chromatid breaks per cell induced by bleomycin were determined. The presence of 13-*cis*-retinoic acid decreased the number of breaks per cell by 13.0 to 59.5% in lymphoblastoid cell lines and by 0 to 57.4% in primary lymphocyte cultures (in the concentration ranges of 10^{-8}–10^{-6} mol/l and of 10^{-8}–10^{-5} mol/l, respectively). Regression analysis showed that there was a statistically significant correlation between the presence of 13-*cis*-retinoic acid and protection against bleomycin-induced clastogenicity. These data give additional information to the knowledge of possible chemopreventive mechanisms of action of 13-*cis*-retinoic acid.
Eur J Cancer, Vol. 29A, No. 1, pp. 137–140, 1993.

INTRODUCTION

VITAMIN A IS important for the normal differentiation of epithelial cells; its presence is required for the physiological secretory function of the epithelial lining in the upper aerodigestive tract. There are indications that retinoids have a preventive effect on carcinogenesis in a variety of epithelial tissues, including the oral mucosa and airways in both animals and humans. Recent investigations have suggested that retinoids act primarily by regulating gene expression [1, 2], and through this mechanism retinoids may modulate the growth of premalignant cells or suppress the progression of premalignant cells to neoplastic lesions.

In clinical trials, 13-*cis*-retinoic acid (isotretinoin), a synthetic retinoid, has been shown to effectively suppress premalignant lesions of the oral cavity [3]. Furthermore, high doses were effective in preventing second primary tumours in patients who had been previously treated for an initial squamous cell carcinoma of the upper aerodigestive tract [4].

Chromosomal fragility is an indicator of genetic instability, and is associated with an increased risk of cancer in syndromes such as Fanconi's anemia, xeroderma pigmentosum, ataxia-telangiectasia, and Bloom's syndrome [5]. In the general popu-

Correspondence to T.C. Hsu.
Z. Trizna and W.K. Hong are at the Department of Medical Oncology (Box 80); Z. Trizna and T.C. Hsu are at the Department of Cell Biology (Box 181); J.J. Lee is at the Department of Biomathematics (Box 237), The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, Texas 77030, U.S.A.; Z. Trizna is also at the Department of Head and Neck Surgery, National Institute of Oncology, Budapest, Rath Gy.u. 7–9., Hungary H-1122; and S.P. Schantz is at the Department of Head and Neck Surgery, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, Room C973, New York, New York 10021, U.S.A.
Revised 7 May 1992; accepted 26 May 1992.