Influence of low pH on cytotoxicity of paclitaxel, mitoxantrone and topotecan

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Summary The extracellular pH (pHe) of solid tumours is often lower than in normal tissues, and this may influence the uptake and/or activity of anti-cancer drugs. The cytotoxicity of mitoxantrone, paclitaxel and topotecan was therefore assessed at low pHe and after manipulation of intracellular pH (pHi) in murine EMT6 and in human MGH-U1 cells. The cytotoxic efficacy of all three agents was reduced at pHe 6.5 as compared with pHe 7.4. The ionophore nigericin and inhibitors of membrane-based ion exchange mechanisms that regulate pHi (S-[N-ethyl-N-isopropyl] amiloride, EIPA; 4,4-diisothiocyanstilbene 2,2-disulphonic acid, DIDS) were used to cause intracellular acidification. Combined use of the cytostatic drugs with pHi modifiers reduced their cytotoxicity under both physiological and low-pHe conditions. The uptake into cells of mitoxantrone (a weak base) was inhibited at pHe 6.5 as compared with pHe 7.4, and smaller effects of low pHe to inhibit uptake of topotecan were also observed. DNA analysis of cell cycle distribution revealed that intracellular acidification, as observed during incubation at low pHe and/or using pHi modifiers, resulted in accumulation of cells in G2 phase, where they may be more resistant to these drugs. Reduced uptake of weak bases (mitoxantrone) at low pHe and altered cell cycle kinetics upon acidification are the postulated causes of reduced cytotoxicity of the agents investigated.

Keywords: pH; paclitaxel; mitoxantrone; topotecan

Solid tumours are known to develop a microenvironment in which the pH (pHe) is often lower than in normal tissues (Wike-Hooley et al, 1984; Vaupel et al, 1989). In contrast, intracellular pH (pHi) as assessed by 31P magnetic resonance spectroscopy is usually maintained at physiological levels in both tumours and normal tissues, as is expected for the survival of constituent cells, although severely hypoxic tumours may have lower pHe values (Vaupel et al, 1994). The maintenance of physiological values of pHi in the face of an acidic pHe depends on the buffering capacity of the cell and on membrane-based ion exchangers, the Na+/H+ antiport and the Na+-dependent HCO3-/Cl- exchange mechanism (Madshus et al, 1988; Boyer et al, 1992). These exchangers may be inhibited respectively by amiloride and its analogues (e.g. 5-[N-ethyl-N-isopropyl] amiloride, EIPA) and by stilbene derivatives (e.g. 4,4-diisothiocyanstilbene 2,2-disulphonic acid, DIDS).

The presence of an H+ gradient across the membrane of tumour cells has implications for chemotherapy. Acidic values of pHe are likely to facilitate the uptake of weak acids, such as melphalan (Skarsgaard et al, 1995), as more of the compound will be in the uncharged form at low values of pHe (Karuri et al, 1993). In contrast, extracellular acidity will inhibit the uptake of weak bases such as doxorubicin (Alabaster et al, 1989). These effects may be modified by agents that dissipate the pH gradient across the membrane (e.g. the K+/H+ exchange ionophore, nigericin) and/or by inhibition of the membrane-based exchange mechanisms that regulate pHi (Parkins et al, 1996).

Paclitaxel, mitoxantrone and topotecan (9-dimethyl-amino-methyl-20-hydroxy-camptothecin) are anti-cancer drugs that are gaining increasing importance in the therapy of solid tumours. In the present paper we assess the influence of acidic pHe on their uptake and/or activity, and the influence on their cytotoxicity of agents that modify the pH gradient across the cell membrane.

Figure 1 Intracellular pH (pHi) measurement using BCECF AM. MGH-U1 and EMT6 cells were incubated in the presence of EIPA (E) and DIDS (D) and/or nigericin (N) for 3 h at pHe 7.4 (□) and pHe 6.5 (■). Columns are mean values, error bars are standard deviations.
MATERIALS AND METHODS

Reagents

Nigericin, DIDS and the buffers Hepes and Bis-Tris were purchased from Sigma (St Louis, MO, USA). EIPA was purchased from Research Biochemical International (Natick, MA, USA). DIDS was dissolved in 50% DMSO. EIPA was dissolved in 10% DMSO and nigericin was dissolved in absolute ethanol. In cell culture experiments, the final concentration of each solvent was < 0.1%. Mitoxantrone (Novantrone) was purchased as the formulation for clinical use from Wyeth-Ayerst Canada (Montreal, PQ, Canada); paclitaxel (Taxol) was purchased as the formulation for clinical use from Bristol-Myers Squibb Canada (Montreal, PQ, Canada); and topotecan was provided by SmithKline Beecham Pharmaceuticals (King of Prussia, PA, USA). BCECF-AM was purchased from Molecular Probes (Eugene, OR, USA).

Cells

Experiments were performed with murine EMT6 cells (obtained originally from Dr R Sutherland, University of Rochester, NY, USA) and the human bladder carcinoma cell line MGH-U1 (obtained originally from Dr G Prout, Massachusetts General Hospital, Boston, MA, USA). Cells were maintained in α-MEM, supplemented with 10% fetal bovine serum (FBS) and 0.1 mg ml⁻¹ kanamycin and were passaged routinely twice a week. Cells were discarded every 3 months and reestablished from frozen stock. They were tested and found to be free of mycoplasma. To prepare medium at different values of pH, 20 mM Hepes or Bis-Tris were added to regular α-MEM and adjusted with 1 N sodium hydroxide or HCl to pH 7.4 or 6.5 respectively. After a 24-h period in the carbon dioxide incubator, the pH of the medium was adjusted to the desired values.

Figure 2  Uptake of mitoxantrone (MX) into EMT6 cells (A), topotecan (TOPO) into EMT6 cells (C), MX into MGH-U1 cells (B) and TOPO into MGH-U1 cells (D). Cells were incubated in the presence of EIPA (E) and DIDS (D) and/or nigericin (N) for 3 h at pH 7.4 (□) and pH 6.5 (■). Columns are mean values, error bars are standard deviations.
Measurement of $pH_i$

Exponentially growing EMT6 and MGH-U1 cells were detached using 0.025% trypsin and 0.01% EDTA, washed and resuspended in α-MEM, and plated on 24 mm x 6 mm glass coverslips ($5 \times 10^4$ cells per coverslip). The coverslips were placed in 60-mm Petri dishes (Nunc, Kamstrup, Denmark) with 5 ml of α-MEM. After 24 h, the medium was replaced with α-MEM at pH 7.4 or 6.5, with or without EIPA (10 μM), DIDS (100 μM) and nigericin (0.3 μM).

Thirty minutes before $pH_i$ measurement, 2 μg ml$^{-1}$ BCECF-AM was added to the samples. After dye loading, the coverslips were rinsed in phosphate-buffered saline (PBS) and the ratio of intracellular fluorescence emission at 525 nm after excitation at 495 nm ($pH_i$ sensitive) to that at 440 nm ($pH_i$ insensitive) was determined.

A fluorescence calibration curve for different $pH_i$ values was established using the ionophore nigericin and solutions containing 140 mM K$^+$, as described elsewhere (Boyer et al, 1992).
Drug uptake

The uptake of mitoxantrone and topotecan into cells was assessed by using flow cytometry. Exponentially growing EMT6 and MGH-U1 cells were detached using 0.025% trypsin and 0.01% EDTA, washed and resuspended in α-MEM, buffered to pH 7.4 or 6.5, containing either the drugs or solvents. After 24 h of incubation, the drug-containing medium was aspirated, cells were washed with PBS (3 × 5 ml), detached using 0.025% trypsin and 0.01% EDTA, washed and resuspended in α-MEM. The cells were counted, serially diluted and plated in triplicate in 60-mm Petri dishes. After approximately 8 days’ incubation, colonies were stained with methylene blue and counted. All experiments were performed three times. The pH of media was determined before and after cell incubation. Dishes in which there was variation of more than 0.1 pH unit from the preset pH values were discarded.

DNA analysis

Because the activity of drugs depends on cell cycle phase distribution, we analysed the DNA content of cells under different conditions using flow cytometry. For DNA analysis, samples were prepared as for cell survival experiments. After a 24-h incubation at pH 7.4 or pH 6.5, in the presence of EIPA (10 μM), DIDS (100 μM) and/or nigericin (0.3 μM) or under control conditions, exponentially growing cells were washed with PBS (3 × 5 ml), and detached using 0.025% trypsin and 0.01% EDTA. After centrifugation, cell pellets were resuspended in 1 ml of PBS containing 50 μg ml⁻¹ propidium iodide and 0.1% Triton X-100, and incubated for 30 min at 37°C. After excitation at 488 nm, fluorescence from 5 × 10⁵ events per sample was collected at 640 nm. DNA histogram analysis was performed using the Phoenix Flow Systems Multicytle software (San Diego, CA, USA). Experiments were repeated three or four times.

RESULTS

Measurement of pH

As shown in Figure 1, only the combined presence of EIPA, DIDS and nigericin decreased the pH significantly in either cell line when exposed to pH 7.4. At pH 6.5, acidification occurred in the absence of modifying agents in MGH-U1 but not in EMT6 cells; addition of EIPA and DIDS acidified EMT6 cells, but led to no further acidification of MGH-U1 cells compared with control. When nigericin was added to MGH-U1 cells exposed at pH 6.5, either alone or in combination with EIPA and DIDS, pH decreased to about pH 6.6; nigericin was less effective when used alone for EMT6 cells, but the combination of three agents acidified EMT6 cells to pH 6.4 after 3 h of incubation. This decrease was time dependent (data not shown).

Uptake of mitoxantrone and topotecan

The uptake of mitoxantrone into cells, as shown in Figure 2A and B, was approximately twofold higher at pH 7.4 than at pH 6.5 (EMT6 cells P < 0.01, MGH-U1 cells P < 0.05). A small increase in cellular accumulation of mitoxantrone at both pH values was observed when the Na⁺/H⁺ antiport and the Na⁺-dependent HCO₃⁻/Cl⁻ exchanger were blocked with 10 μM EIPA and 100 μM DIDS respectively. When cells were incubated in the presence of nigericin, with or without EIPA and DIDS, uptake of mitoxantrone was slightly greater at pH 7.4, and similar at pH 6.5, to uptake under control conditions.

The uptake of topotecan into EMT6 and MGH-U1 cells is shown in Figure 2C and D. Treatment with EIPA and DIDS in

Figure 5 Influence of pH modifiers on cytotoxicity of mitoxantrone (MX) (A), paclitaxel (PX) (B) and topotecan (TOPO) (C) under physiological and low pH conditions. EMT6 cells were incubated for 24 h at pH 7.4 (C) and pH 6.5 (B) in the presence of mitoxantrone [10 nM], paclitaxel [100 nM] and topotecan [3 μM], with or without EIPA (E: 10 μM), DIDS (D: 100 μM) and/or nigericin (N: 0.3 μM). Columns are mean values, error bars are standard deviations.
combination did not alter the uptake of topotecan at pH 7.4 or 6.5. Nigericin increased significantly the uptake of topotecan at pH 7.4 but only minimally at pH 6.5. When all three pH modifiers were added, drug uptake decreased to control levels at pH 7.4 and to values lower than under control conditions at pH 6.5.

Cell survival experiments

Greater cytotoxicity for each drug was observed at pH 7.4 than at pH 6.5 in both cell lines investigated (Figures 3 and 4). The effects of modifiers of pH on cytotoxicity are shown for EMT6 cells in Figure 5. Similar results were found for MGH-U1 cells (data not shown).

The pH concentration equivalent to LD₅₀ was chosen for experiments using pH modifiers, as it should be possible to determine both increases or decreases in cytotoxicity within one experiment. The pH modifiers alone (EIPA 10 μM, DIDS 100 μM and nigericin 0.3 μM) did not exert significant cytotoxicity. Only the combination of all three agents at pH 6.5 caused significant cell kill, as has been described previously (Rotin et al., 1987). The cytotoxicity of mitoxantrone was reduced substantially in the presence of EIPA and DIDS. When incubated with nigericin, the cytotoxicity of mitoxantrone was decreased both at pH 7.4 and 6.5 by a factor of 2. When all three pH modifiers were incubated with mitoxantrone, the observed cytotoxic effects were not different to the pH modifiers used alone. The results obtained with paclitaxel and topotecan are very similar to mitoxantrone, showing decreased cytotoxic potency of the drugs when combined with EIPA and DIDS or nigericin (Figure 5).

DNA analysis

The distributions of cells in different phases of the cell cycle after incubation for 24 h at pH 7.4 or 6.5 in the presence or absence of modifiers of pH₅₀ are shown in the Table. At pH 7.4, untreated exponentially growing EMT6 and MGH-U1 cells show a nearly equal distribution of cells in G₁ and S/G₂, cell cycle phases. In the presence of either nigericin or EIPA and DIDS, the number of cells in G₁ phase increases to about 60%. Combination of all three pH modifiers increases the number of EMT6 and MGH-U1 cells in G₁ phase to approximately 72% and 80% respectively. The number of cells in G₂ phase decreases in the presence of nigericin to approximately half of the number of cells treated with EIPA and DIDS, or control. At pH 6.5, untreated EMT6 cells have approximately 54% of cells in G₂ phase, increasing to approximately 80% when the cells were incubated in the presence of nigericin. Conditions of pH 6.5, with or without pH₅₀ modulators, had a similar effect on the cell cycle distribution of MGH-U1 cells (Table).

DISCUSSION

The present study addresses two questions: (a) whether low pH (6.5), as can be found in acidic regions of many solid tumours, influences the cytotoxicity of mitoxantrone, paclitaxel and topotecan; and (b) the effects of intracellular acidification using EIPA, DIDS and nigericin, on the cytotoxic potency of these drugs. Our results show that the in vitro cytotoxic effects of all three drugs for EMT6 and MGH-U1 cells are reduced at low pH₅₀ and that their effects are not enhanced by agents that induce intracellular acidification.

Our findings support the results of Jahde et al (1990), who reported a decrease in cytotoxicity of mitoxantrone at low pH₅₀. Our data on mitoxantrone uptake show that there is a substantial decrease in cellular accumulation at pH 6.5 when compared with pH 7.4, which is consistent with the observed decrease in mitoxantrone cytotoxicity at pH 6.5. We observed a slight increase in cellular accumulation of mitoxantrone when pH₅₀ modifiers EIPA and DIDS were added. This effect is in contrast to the results of a study of the effects of EIPA and DIDS on accumulation of doxorubicin (Asaumi et al., 1995). One postulated mechanism for decreased cytotoxicity of mitoxantrone at low pH₅₀ is the protonation status of mitoxantrone, a weak base, at different pH₅₀. At physiological pH₅₀ (approximately 7.4), a larger proportion of drug molecules would be uncharged, thus facilitating diffusion into cells (Karuri et al., 1993). At low pH₅₀ (< 7.0), the proportion of charged drug molecules would increase, resulting in decreased drug diffusion into cells.

Paclitaxel is not fluorescent, and we did not measure the uptake of this drug as a function of pH₅₀. Owing to its complex structure, with both acidic and basic domains (Huizing et al., 1995), the total charge of the paclitaxel molecule is unlikely to be a simple function of pH₅₀, and it is therefore difficult to predict the effect of pH on drug uptake.

Topotecan, a topoisoerase I inhibitor, exists as a lactone species, which is considered to be the bioactive form, and as a carboxylate species, which represents the bioinactive form of the drug (Potmesil, 1994). Hydrolysis of the lactone form to the carboxylate species is very rapid under physiological conditions (pH approximately 7, 37°C); Owing to its positive charge, the

| Sample                     | EMT6 cells | MGH-U1 cells |
|----------------------------|------------|--------------|
|                            | pH 7.4     | pH 6.5       | pH 7.4     | pH 6.5       |
|                            | G₁         | S            | G₂         | G₁         | S            | G₂         | G₁         | S            | G₂         |
| Untreated cells            | 49.8       | 40.1         | 10.1       | 53.6       | 35.3         | 11.1       | 51.7       | 39.0         | 9.3         | 54.2       | 34.5         | 11.3       |
| EIPA + DIDS                | 55.1       | 31.6         | 13.3       | 57.2       | 33.3         | 9.5        | 58.2       | 30.8         | 11.0        | 57.6       | 32.1         | 10.3       |
| Nigericin                  | 57.3       | 35.6         | 7.1        | 72.5       | 21.1         | 6.4        | 58.7       | 34.8         | 6.5         | 71.2       | 20.6         | 8.2        |
| EIPA + DIDS + nigericin    | 71.5       | 22.3         | 6.2        | 80.1       | 13.3         | 5.7        | 79.4       | 14.3         | 6.3         | 79.9       | 11.4         | 8.7        |

Means are from three or four experiments.
carboxylate form has reduced ability to diffuse passively into cells
and to interact with topoisomerase I (Hertzberg et al, 1989). At
low pH, hydrolysis to carboxylate is expected to be slower, with
a higher yield of the active drug form, and possibly higher cytotoxic-
ity in cell survival experiments. Under the experimental condi-
tions investigated, however, the uptake of topotecan was enhanced
when pH was at physiological values and intracellular acidifica-
tion was induced with nigericin. The increased uptake of topotecan
in the presence of nigericin, however, was not accompanied by an
equivalent increase in cytotoxicity. As nigericin is known to
increase the intracellular H+ concentration by facilitating K+/H+
exchange across the cell membrane, the bioactivity of topotecan
might be influenced by changes in cellular ions or by some other,
non-specific effects related to changes in pHn.

Intracellular acidification has been reported to inhibit cell-cycle
progression, leading to enrichment of cells in the G1 phase of the
cell cycle (Musgrove et al, 1987). This effect was observed more
rapidly in EMT6 than in MGH-U1 cells (Table). It has been shown
that HeLa and SQ20B cells are most sensitive to paclitaxel and
docetaxel in S-phase and most resistant in G1/G0 phase (Hennequin et al, 1996), thus providing a plausible explanation for the
approximately threefold decrease in cytotoxicity when paclita-
taxel was incubated with cells at low pHn with or without pHn
modifiers. This effect probably contributes also to resistance to
mitoxantrone (Sundman-Engberg et al, 1996) and topotecan, as
most cytostatic drugs are more active against cycling cells.

Our results suggest that low values of pHn might contribute to
resistance of solid tumours to mitoxantrone, paclitaxel and
topotecan. As values of pHn in certain areas of solid tumours are
known to be acidic, cells from these regions are more likely to
survive and repopulate a tumour after chemotherapy with agents
showing decreased cytotoxic potency under acidic conditions. Our
results do not suggest that manipulation of the pH gradient across
the cell membrane with EIPA, DIDS and/or nigericin will increase
the therapeutic effectiveness of these drugs.

ABBREVIATIONS
EIPA, 5-(N-ethyl-N-isopropyl) amiloride; DIDS, 4,4-diisothio-
cyanstilbene 2,2-disulphonic acid; a-MEM, alpha-minimum essential
medium; BCECF-AM, 2',7'-bis-(2-carboxyethyl)-5-(and 6)-carboxy-
fluorescein acetoxyethyl ester; pH, extracellular pH; pHn, intracellular
pH; Hepes, N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid;
Bis-Tris, bis(2-hydroxyethyl)limino-tris(hydroxymethyl) methane.

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