PERGAMON

European Journal of Cancer 39 (2003) 838–846

European Journal of Cancer

www.ejconline.com

The radiosensitising effect of gemcitabine and the influence of the rescue agent amifostine *in vitro*

B. Pauwels[a,*], A.E.C. Korst[a], C.M.J. de Pooter[b], H.A.J. Lambrechts[a], G.G.O. Pattyn[a], F. Lardon[a], J.B. Vermorken[a]

[a]*Laboratory of Cancer Research and Clinical Oncology, Department of Medical Oncology, University of Antwerp (UIA/UZA), Universiteitsplein 1, 2610 Wilrijk, Belgium*

[b]*Department of Radiotherapy, St Augustinus Hospital, Antwerp, Belgium*

Received 15 August 2002; received in revised form 8 November 2002; accepted 19 December 2002

Abstract

In this study, the radiosensitising effect of different concentrations of gemcitabine and the combination of gemcitabine/radiotherapy with the rescue agent amifostine was investigated in different human tumour cell lines. The cells were treated with gemcitabine (0–8 nM) for 24 h prior to radiation (0–8 Gy). Amifostine (ami) and alkaline phosphatase (AP) were added 30 min before radiation. Cell survival was determined 7 or 8 days after radiation treatment by the sulforhodamine B (SRB) test. For ECV304 cells, the dose enhancement factor (DEF) varied from 1.39 to 2.98 after treatment with 1–6 nM gemcitabine. FaDu, H292, A549 and CAL-27 seemed to be less sensitive, with DEFs ranging from 1.02 to 2.67. These cells were also less sensitive to the cytotoxic effects of single-agent gemcitabine. Amifostine with AP clearly showed a protective effect in combination with gemcitabine/radiotherapy. In H292 cells, the protection factor (PF) of amifostine after treatment with gemcitabine and radiotherapy varied from 1.64 to 1.86. In ECV304 cells, the PF varied from 2.20 to 2.29. In conclusion, a clear concentration- and cell line-dependent radiosensitising effect of gemcitabine was observed in all cell lines. Amifostine with AP showed protection against the radiosensitising effect of gemcitabine. If the protection *in vivo* indeed occurs selectively in normal tissues, then amifostine could prevent or strongly minimise the increased toxicity resulting from the radiosensitising effect of the combination of gemcitabine and radiotherapy, without influencing the antitumour effect.

© 2003 Elsevier Science Ltd. All rights reserved.

Keywords: Radiation; Gemcitabine; Radiosensitisation; Amifostine; Cytoprotection

1. Introduction

Several biological factors are known to influence the efficacy of radiotherapy (RT) when given as a single treatment modality to patients with locally or regionally advanced cancer. Increased attention is being given to combining radiotherapy with other treatment modalities, especially chemotherapy, because cytotoxic agents act on both the primary tumour and disseminated cancer cells. Some chemotherapeutic drugs destroy tumour cells by their own cytotoxic action and also enhance the effects of radiotherapy. Because of this radiosensitisation, the results of the combined use of radiotherapy with these cytotoxic drugs have been encouraging, both in terms of increased local tumour control and decreased distant failures.

Gemcitabine (2′,2′-difluorodeoxycytidine, dFdC) is a synthetic pyrimidine nucleoside analogue that has a structure very similar to that of deoxycytidine and cytosine arabinoside (Ara-C) [1]. In clinical use, gemcitabine is active against a variety of solid tumours such as cancers of the pancreas, lung, head and neck, bladder, breast and ovary. It is activated intracellularly by deoxycytidine kinase, which adds multiple phosphate groups to the 5′ position of the ribose group. The diphosphate and triphosphate forms of the drug play an important role in the inhibition of DNA synthesis. At the same time, gemcitabine has several self-potentiation mechanisms that serve to increase intracellular levels of the active compound [1,2].

* Corresponding author. Tel.: +32-382-02576; fax: +32-382-02248.

E-mail address: bea.pauwels@ua.ac.be (B. Pauwels).

0959-8049/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
PII: S0959-8049(03)00002-9

In addition to its cytotoxic effect, gemcitabine is a potent radiosensitiser of rodent and human tumour cells, including pancreatic tumours, non-small cell lung cancer, head and neck cancer, colorectal, breast and ovarian cancers [3–13]. The mechanism of radiosensitisation is not yet known, it has been hypothesised that gemcitabine-induced deoxyadenosine triphosphate (dATP) depletion or the accumulation of the cells in early S phase may play a role in enhancing radiosensitivity [5,7,10,13,14]. In addition, the ability of cells to progress into S phase after treatment with gemcitabine and radiotherapy may be a key factor [11]. Recently, it has been shown that gemcitabine can sensitise cells to radiation by specific interference with the homologous repair (HR) pathway [15].

Although combined-modality therapy may lead to an improved therapeutic outcome, it can also result in increased normal tissue toxicity. Since the combination of gemcitabine and radiotherapy has been associated with a marked increase in toxic effects in clinical studies [16–23], it seemed reasonable to postulate that a cytoprotective agent might reduce the toxicity of this combination. Amifostine (ami) is one of the most promising modulating agents because of its ability to selectively protect a broad range of normal tissues against a variety of cytotoxic therapies including alkylating agents, platinum agents, anthracyclines, taxanes and irradiation. In these studies, amifostine did not reduce the cytotoxic effect of irradiation or chemotherapy on tumours [24].

Amifostine is a prodrug, which is dephosphorylated by the membrane-bound enzyme alkaline phosphatase (AP) to the free thiol metabolite, WR1065. WR1065 is the active form of the drug and is responsible for the protective effects of amifostine [25,26]. Once inside the cell, WR1065 protects against chemotherapy- and radiotherapy-induced damage by scavenging free radicals, donating hydrogen ions to free radicals, depleting oxygen and directly binding and inactivating cytotoxic drugs, thereby either avoiding or repairing DNA damage. The selective protection of non-tumour tissues by amifostine is generally thought to be related to the selective formation and uptake of WR1065 in normal tissues [24,27–30]. AP, the membrane-bound enzyme responsible for the dephosphorylation of amifostine to the WR1065 metabolite, is present in higher concentrations in normal compared with tumour tissues [30]. Because amifostine protects a broad range of normal tissues from the toxic effects of both chemotherapy and radiotherapy, it could be very useful when applying radiochemotherapy combinations. In fact, in patients with lung or head and neck cancer receiving the combined approach, amifostine has already been shown to reduce severe toxicities [24].

Since the combined use of gemcitabine and radiotherapy has been shown to be not only active, but also rather toxic, it seemed desirable to investigate whether amifostine might be of value in this combination to protect normal tissues, but not tumour tissues, thereby improving its therapeutic index. To form a basis for this research, we investigated the radiosensitising effect of gemcitabine in five human cell lines and studied the combination with amifostine. The selectivity of the protective effect can only be studied *in vivo*, because the protection is based on preferential formation and uptake of the active metabolite in normal tissues. However, since the selectivity of the protection by amifostine has already been confirmed in several clinical studies, the most important question is whether amifostine can protect against the combination of gemcitabine and radiotherapy. To address this question, we treated cells with amifostine combined with AP and therefore, circumvented the selectivity of the protective effect. Since, in this case, the cell type does not play a role in this protection, we used, for practical reasons, our tumour cell lines to investigate a possible protective effect of amifostine in combination with gemcitabine and radiation.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute (RPMI), Medium 199, fetal calf serum and the medium supplements L-glutamine and sodium pyruvate were all purchased from Invitrogen (Merelbeke, Belgium). Sulforhodamine B (SRB) was from ICN (Asse, Belgium). Gemcitabine was purchased from Eli Lilly (Indianapolis, USA). Alkaline phosphatase (AP, Type VII-S: from bovine intestinal mucosa) was obtained from Sigma (Bornem, Belgium). Amifostine (S-2-(3-aminopropylamino)ethylphosphorothioic acid) was a kind gift of MedImmune (Gaithersburg, USA).

2.2. Cell lines

The cell lines used in this study were ECV304, a human epidermoid bladder cancer cell line, H292, a human mucoepidermoid lung cancer cell line, A549, a human squamous lung cancer cell line, CAL-27, a human squamous cell carcinoma cell line of the tongue and FaDu, a human squamous cell carcinoma cell line of the pharynx. H292 and A549 were cultured in RPMI-1640 medium, supplemented with L-glutamine, sodium pyruvate and 10% fetal calf serum. ECV304 was cultured in Medium-199 supplemented with 10% fetal calf serum. CAL-27 and FaDu were cultured in DMEM medium, supplemented with L-glutamine and 10% fetal calf serum. Cultures were maintained in exponential growth in a humidified atmosphere at 37 °C under 5% CO_2/95% air.

2.3. Cell survival after treatment with gemcitabine and radiation

The SRB test is a suitable test system for *in vitro* radiosensitivity testing, which in the presently used cell lines has been shown to be comparable in outcome with the clonogenic assay, when cells are allowed to undergo at least 6 doubling times after radiation treatment [31]. Therefore, in our experiments ECV304, H292, A549 and FaDu cells were incubated for 7 days and CAL-27 cells for 8 days after radiation treatment, before determination of the survival by the SRB assay. Optimal seeding densities were determined for each cell line to assure exponential growth during the assay.

Cells were harvested from exponential phase cultures by trypsinisation, counted and plated in 48-well plates. In order to assure exponential growth during the experiments, seeding densities were 100 cells per well for ECV304, H292 and A549, 160 cells per well for FaDu and for CAL-27 the seeding density was 300 cells per well. Following plating and a 24-h recovery period, cells were treated with gemcitabine (0–8 nM) dissolved in phosphate-buffered saline (PBS) 24 h before radiation. PBS was added to control cells. Each concentration was tested six times within the same experiment. After irradiation at room temperature over a dose range of 0–8 Gy, using a ^{60}Co source (Alcyon, St Augustinus hospital, Antwerp, Belgium), cells were washed with drug-free medium. After 7 or 8 days, the survival was determined by the SRB assay. For determination of cell survival after treatment with gemcitabine alone, the SRB assay was performed 4 days after the start of treatment.

The SRB assay was performed according to the method of Skehan and colleagues and Papazisis and colleagues, with minor modifications [32,33]. Culture medium was aspirated prior to fixation of the cells by the addition of 200 μl 10% cold trichloroacetic acid. After 1 h incubation at 4 °C, cells were washed five times with deionised water. Then the cells were stained with 200 μl 0.1% SRB dissolved in 1% acetic acid for at least 15 min and subsequently washed four times with 1% acetic acid to remove unbound stain. The plates were left to dry at room temperature and bound protein stain was solubilised with 200 μl 10 mM unbuffered TRIS base (tris(hydroxymethyl)aminomethane) and transferred to 96-well plates for reading the optical density (OD) at 540 nm (Biorad 550 microplate reader, Nazareth, Belgium).

2.4. Protective effect of amifostine, alkaline phosphatase or both in combination with radiation treatment

The protective effect of amifostine and AP alone or the combination of both with radiation was investigated in H292 cells. Cells were plated in 48-well plates as described above. 30 min before radiation treatment, cells were incubated with 3.5 mM amifostine or 7.5 units AP or both 3.5 mM amifostine and 7.5 units AP. PBS was added to control cells. The cells were irradiated (0–8 Gy) and washed with fresh medium immediately after radiation. After 7 days, the survival was determined by the SRB assay.

2.5. Protective effect of amifostine with AP in combination with gemcitabine and radiation treatment

The cytoprotective effect of amifostine in combination with gemcitabine and radiation was investigated in ECV304 and H292 cells. Cells were plated in 48-well plates as described above. After a 24 h treatment with radiosensitising concentrations of gemcitabine (4 and 8 nM gemcitabine for H292, 2 and 4 nM gemcitabine for ECV304), the cells were treated with 3.5 mM amifostine and 7.5 units AP in PBS 30 min before the radiation. PBS was added to control cells. The cells were irradiated and washed with drug-free medium immediately after radiation. After 7 days, their survival was determined by the SRB assay.

2.6. Statistical methods

The survival rates were calculated by: mean OD of treated cells/mean OD of control cells×100%. The radiation survival curves were fitted according to the linear-quadratic model: survival = $\exp(-\alpha D-\beta D^2)$, using Winnonlin (Pharsight, USA).

The following parameters were calculated: the linear component α, which represents single-hit killing kinetics, sublethal damage and dominates at low radiation doses; the quadratic component β, which represents double-strand breaks at DNA level, lethal damage and causes the curve to bend at higher doses and ID50, the radiation dose causing 50% growth inhibition. The radiosensitising effect was represented by the dose enhancement factor (DEF): ID50 (−dFdC)/ID50(+dFdC). The protection factor (PF) was calculated by ID50(+ami+AP)/ID50(−ami−AP).

Unless otherwise indicated, all of the data are presented as the mean±standard deviation. All experiments were performed at least three times. A two-sample *t*-test was used to investigate significant differences between ID50 values, α and β values.

True radiosensitisation can be defined as a synergistic interaction between gemcitabine and radiation. For the determination of synergism, the combination index (CI) was calculated by the Chou–Talalay equation [34,35], using CalcuSyn (Biosoft, USA and UK). The general equation for the classic isobologram is given by:

$$\mathrm{CI} = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} \quad \text{(A)}$$

where $(D_x)_1$ and $(D_x)_2$ in the denominators are the doses (or concentrations) for D_1 (gemcitabine) and D_2 (radiation) alone that give x% inhibition, whereas $(D)_1$ and $(D)_2$ in

the nominators are the doses of gemcitabine and radiation in combination that also inhibit x% (i.e. isoeffect). CI < 1, CI = 1 and CI > 1 indicate synergism, additive effect and antagonism, respectively.

The $(D_x)_1$ or $(D_x)_2$ (for gemcitabine and radiation) can be readily calculated from the median-effect equation of Chou:

$$D_x = D_m\left(\frac{f_a}{(1-f_a)}\right)^{1/m}$$

where D_m is the median-effect dose (ID50 or IC50) that is obtained from the anti-log of the X-intercept of the median effect plot, X-log (D) versus $Y = \log f_a/(1-f_a$ or $D_m = 10^{-(Y\text{-intercept})/m}$, and m is the slope of the median effect plot.

A CI value between 0.9 and 1.1 indicates only additivity. Moderate synergism is depicted by CI values between 0.7 and 0.9, synergism by CI values below 0.7.

3. Results

3.1. Radiosensitisation by gemcitabine

A clear concentration-dependent radiosensitising effect of gemcitabine was observed in ECV304, FaDu, H292, A549 and CAL-27 cells (Fig. 1). ID50 values and DEFs for the different gemcitabine concentrations are summarised in Table 1. The degree of radioenhancement seemed to be cell line-dependent. For ECV304 cells, the DEF after treatment with 1 nM gemcitabine was 1.39, while this concentration had no radiosensitising effect in FaDu, H292, A549 and CAL-27 cells; DEFs were 1.02, 1.09, 1.02 and 1.06, respectively. The CI analysis showed that after treatment during 24 h with gemcitabine before the radiation treatment, there was synergism in ECV304 cells with gemcitabine concentrations of 2 nM or higher ($CI \leqslant 0.65$), with concentrations 4 nM or higher in A549

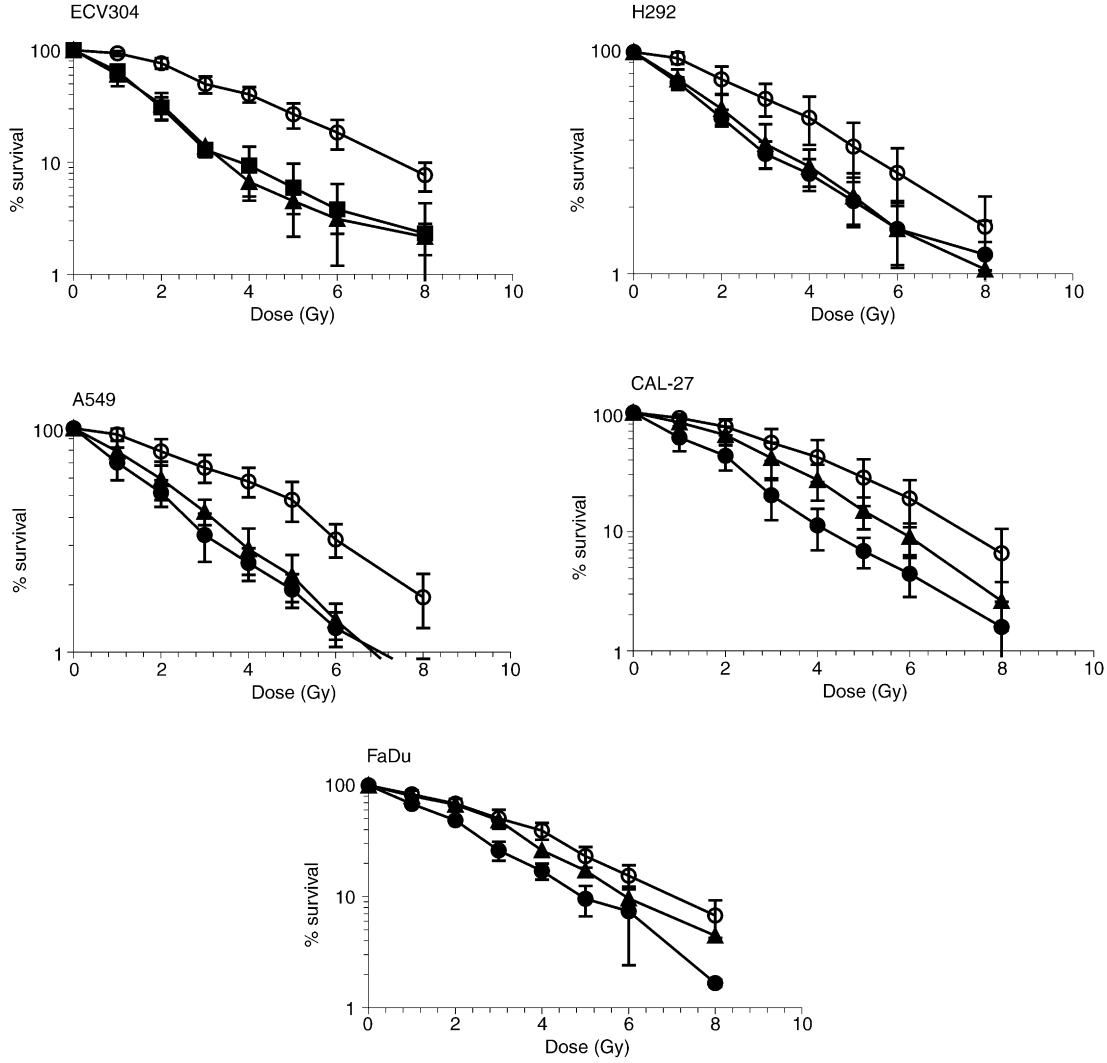

Fig. 1. Radiation dose–response curves of ECV304, H292, A549, CAL-27 and FaDu cells after treatment with 0 (○), 2 (■), 4 (▲) and 6 (●) nM gemcitabine (dFdC) during 24 h before radiation (RT) (survival±S.D.).

Table 1
Sensitivity of the cell lines to gemcitabine (IC50) and radiotherapy (ID50) alone and the radiosensitising effect of gemcitabine represented by dose enhancement factors (mean±S.D.)

Cell line	IC50	ID50	DEF 1 nM	DEF 2 nM	DEF 4 nM	DEF 6 nM	DEF 8 nM
ECV304	3.05±0.49	3.38±0.41	1.39±0.06	2.42±0.24	3.05±0.37	2.98±0.91	n.a.
FaDu	4.90±0.46	3.09±0.39	1.02±0.04	1.23±0.17	1.79±0.25	2.34±0.55	n.a.
H292	7.99±0.77	4.00±0.71	1.09±0.10	1.20±0.20	1.57±0.21	2.08±0.53	2.67±0.63
A549	9.01±0.89	4.51±0.70	1.02±0.04	1.20±0.17	1.90±0.51	2.39±0.57	2.58±0.49
CAL-27	8.92±1.59	3.65±0.85	1.06±0.07	1.09±0.04	1.48±0.21	1.98±0.07	2.52±0.22

IC50/ID50, dose gemcitabine/radiation (dFdC/RT) causing 50% growth inhibition; DEF, dose enhancement factor; n.a., not analysed; S.D., standard deviation.

cells (CI⩽0.70) and FaDu (CI⩽0.50) and moderate synergism with 6 nM or higher in H292 cells (CI⩽0.88) and CAL-27 cells (CI⩽0.72). The radiosensitising effect seemed to correlate with the sensitivity of the cell line to the cytotoxicity effects of gemcitabine. The dose–response curves after treatment with gemcitabine alone and IC50 values are summarised in Fig. 2 and Table 1. FaDu, H292, A549 and CAL-27 cells were less sensitive to gemcitabine than ECV304 cells.

The radiosensitising effect of gemcitabine was observed at the initial part of the dose response curves. This was shown by an increasing α value of the linear quadratic model ranging from 0.08 to 0.68 for ECV304 cells (0–6 nM gemcitabine) and from 0.10 to 0.46, 0.07 to 0.43 and from 0.06 to 0.38 for H292, A549 and CAL-27 (0–8 nM gemcitabine), respectively (Fig. 3). The increase of the α value was most pronounced in the cell line that was most sensitive to gemcitabine, ECV304. In this cell line, the increase in the α value was already statistically significant at a gemcitabine concentration of 1 nM ($P \leqslant 0.05$), while in the other cell lines the increase was significant from 4 nM for H292, A549 and FaDu cells. In CAL-27 cells, the increase in α value was significant from 8 nM.

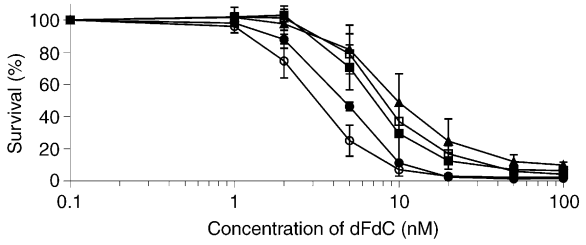

Fig. 2. Dose–response curves of ECV304 (○), FaDu (●), H292 (■), A549 (▲) and CAL-27 (□) after treatment with gemcitabine (dFdC)

3.2. Protection of amifostine, alkaline phosphatase or both with radiation treatment

Amifostine or AP showed no protection against radiation, Protection Factors (PFs) were 1.04 and 0.94, respectively (Fig. 4). The ID50 values after treatment with amifostine or AP were not significantly different from the values after treatment with radiation alone. Amifostine clearly showed a radioprotective effect in our experiments when AP was added, which resulted in a PF of 1.88. The ID50 after treatment with amifostine and AP was significantly higher than the ID50 after radiation alone ($P \leqslant 0.05$). This means that *in vitro* the addition of AP is necessary for the protective effects of amifostine.

3.3. Protection of amifostine with AP in combination with gemcitabine and radiation

Amifostine with AP showed no protection against the cytotoxicity of gemcitabine alone (data not shown), but

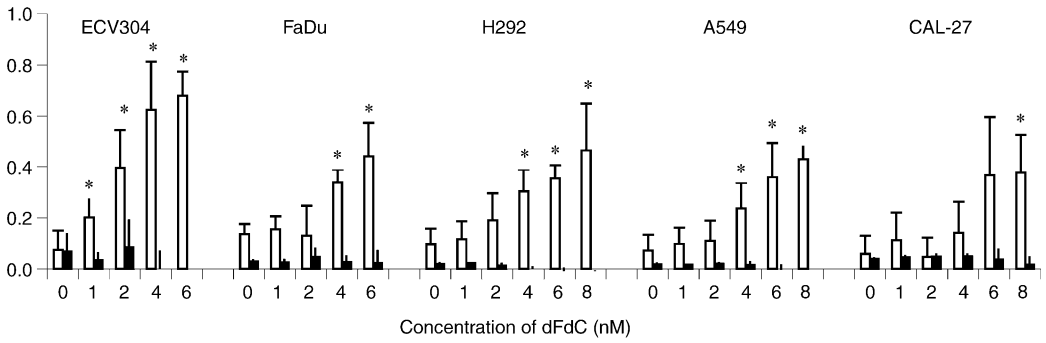

Fig. 3. Influence of the radiosensitising effect of dFdC on α (□) and β (■) in ECV304, H292, A549, CAL-27 and FaDu cells (mean±S.D.). *Student *t*-test, $P \leqslant 0.05$.

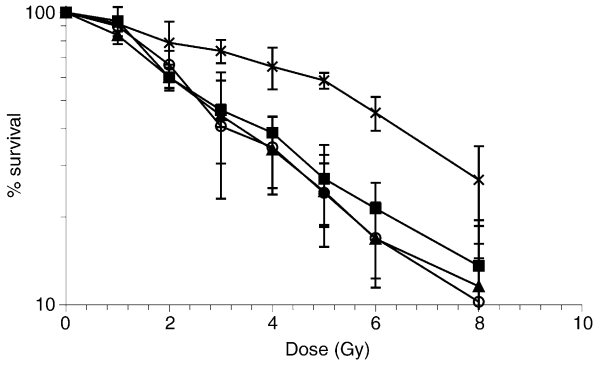

Fig. 4. Radiation dose–response curve of H292 cells after treatment with radiation (RT) (○), RT and amifostine (ami) (■), RT and alkaline phosphatase (AP) (▲) and RT with ami and AP (×) (survival±S.D.). Incubation time of ami and AP is 30 min before the RT.

Table 2
Protection factors of amifostine (ami) with alkaline phosphatase (AP) after treatment with radiotherapy or the combination of gemcitabine (dFdC) and radiotherapy in H292 and ECV304 cells (mean±S.D.)

H292	DEF	PF (−dFdC)	PF (+dFdC)
4 nM dFdC	1.78±0.44	1.42±0.08	1.64±0.15
8 nM dFdC	2.57±0.25	1.54±0.04	1.86±0.04
ECV304	DEF	PF (−dFdC)	PF (+dFdC)
2 nM dFdC	2.41±049	1.72±0.23	2.20±0.21
4 nM dFdC	2.97±0.33	1.91±0.07	2.29±0.10

PF, Protection Factor; S.D., standard deviation.

in combination with radiation treatment and gemcitabine, amifostine with AP clearly reduced the cytotoxicity (Fig. 5). The PFs and DEFs are described in Table 2. In H292 cells, the radiation dose response curve after treatment with 4 nM gemcitabine, radiation and amifostine with AP was not significantly different from the curve after treatment with radiation alone, which means that the protective effect of amifostine was to the same extent as the radiosensitising effect of gemcitabine. In ECV304 cells, the same was observed with 2 nM gemcitabine. With higher concentrations of gemcitabine (8 nM in H292 cells and 4 nM in ECV304 cells), which resulted in higher radiosensitisation, amifostine could not completely negate the radiosensitising effect, although the difference between the curves after treatment with gemcitabine, radiation and amifostine with AP and the curve after treatment with radiation alone was still not significant.

4. Discussion

As a result of an increasing interest in chemoradiation in the clinic, there is a growing need for preclinical research on the interactions between radiation and chemotherapy, especially on radiosensitisers like gemcitabine. Our *in vitro* model offers the opportunity for

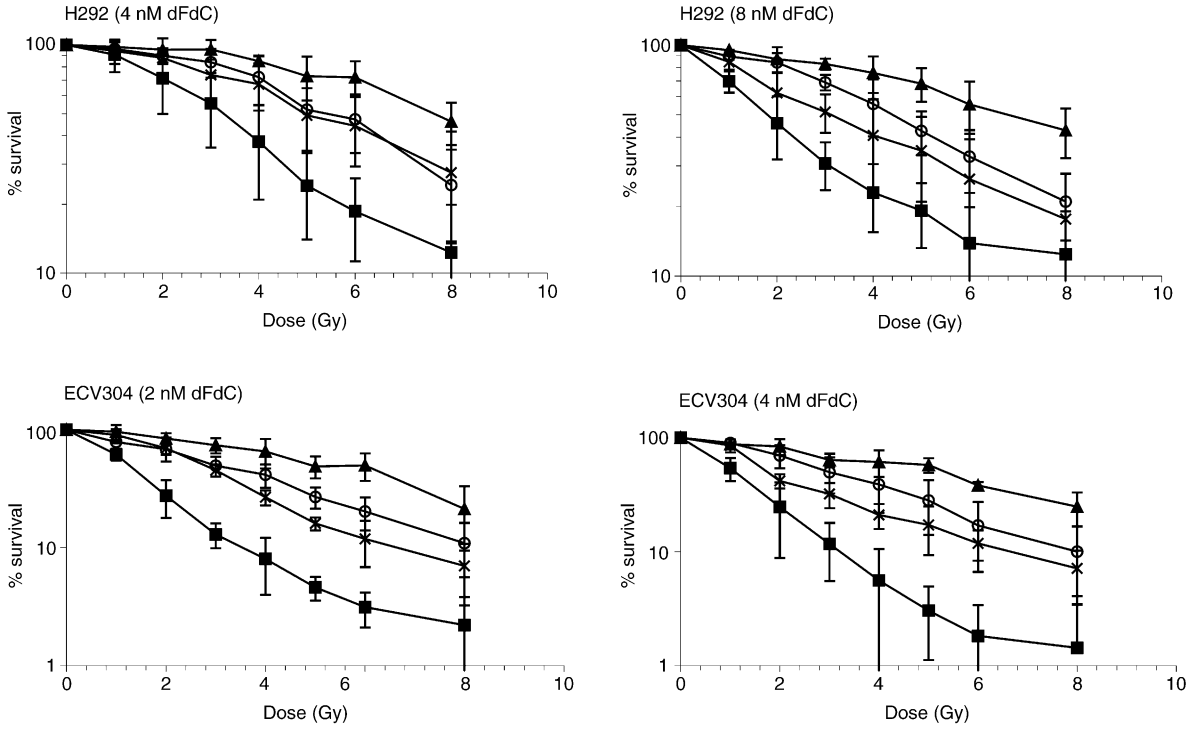

Fig. 5. Radiation dose–response curves of ECV304 and H292 cells after treatment with radiation (RT) (○), RT and gemcitabine (dFdC) (■), RT and ami/AP (▲) and RT, dFdC and ami/AP (×) (survival±S.D.). Incubation time of dFdC is 24 h before RT, with ami and AP 30 min before RT.

more extensive *in vitro* research on such combinations. In the current study, a clear concentration-dependent radiosensitising effect of gemcitabine was observed in several human cell lines representing epithelial tumours.

Several investigators have described the radiosensitising effect of gemcitabine *in vitro* [5–9,11–14], but the mechanism of this radiosensitisation remains unknown. A limiting factor in many studies is the restricted number of cell lines investigated, which hampers the ability to draw firm conclusions regarding which factors play a role in this interaction. Our *in vitro* model broadens the ability to test the combination of gemcitabine and radiotherapy, by the use of the SRB test, a colorimetric assay to determine the survival after treatment with gemcitabine and/or radiation. We have previously shown that the SRB assay is comparable in outcome with the clonogenic assay for radiosensitivity testing in the cell lines used [31].

The present study indeed demonstrates that the radiosensitising effect of gemcitabine can be easily studied using the SRB assay. We investigated the radiosensitising effect in five cell lines, using five different concentrations of gemcitabine. Our data demonstrate that gemcitabine increases the radiosensitivity of ECV304, H292, A549, CAL-27 and FaDu cells *in vitro* when the cells are treated for 24 h before radiation. The enhancement is concentration-dependent, with an increasing DEF with higher concentrations of gemcitabine.

Our DEFs are comparable to data in the literature using other cell lines, such as a squamous carcinoma cell line of the oropharynx and of the uterine cervix [10], human head and neck cancer cell lines [12], glioblastoma cell lines [11], pancreatic cancer cells [8], cell lines of colon and lung [6,7,9,13]. In most studies, the concentration of gemcitabine needed to achieve a radiosensitising effect was higher than that used in our study. This seems to be dependent on the sensitivity of the cell lines employed to gemcitabine. In our experiments, the radiosensitising effect was cell line-dependent and seemed to correlate with the sensitivity of the cell line to the cytotoxic effects of gemcitabine (Fig. 2, Table 1). However, more cell lines with a broader range of IC50 values should be investigated to confirm this relationship. Rosier and colleagues found that radioenhancement was associated with a modest increase in the α term of the linear-quadratic model [12]. We confirmed that gemcitabine has an effect on the α value, which in our study, was most pronounced in ECV304 cells. In the other cell lines, higher concentrations of gemcitabine were needed before statistically significant increases in the α value were documented.

Our *in vitro* study will be expanded to form an important basis for more extensive research on the cell line and schedule dependency of the radiosensitising effect of gemcitabine and the factors playing a role in this interaction.

Although the combined use of radiotherapy with gemcitabine in the clinic has been encouraging, it has been associated with increased toxicity so that standard doses cannot be administered [17–23]. Therefore, maximal radiosensitisation in tumour cells is not by definition the best treatment schedule because of high toxicity in normal tissues. Thus, optimisation of the combination of gemcitabine and radiotherapy needs to focus also on limiting the toxicity. It can be postulated that cytoprotective agents might decrease the toxic effects and provide an improved therapeutic index for the combined treatment.

Amifostine is a promising modulating agent, which selectively protects a broad range of normal tissues against a variety of cytotoxic therapies, including several chemotherapeutic agents and radiotherapy. Amifostine is a prodrug which is dephosphorylated by the membrane-bound enzyme AP to the free thiol metabolite, WR1065. The selectivity of the protective effect is thought to be based on the selective formation and uptake of WR1065 in normal cells as opposed to tumour cells. Amifostine has been evaluated in combination with a variety of different chemotherapy regimens in patients with different types of solid tumours and haematological malignancies [24]. In addition, the radioprotective effects of amifostine have been analysed in patients receiving pelvic, thoracic and head and neck irradiation in a number of comparative trials [24]. Most trials have demonstrated benefit from the addition of amifostine. No evidence of tumour protection so far has been reported in clinical trials [24]. Amifostine might be a promising modulating agent for chemoradiation regimens, because of its broad spectrum of cytoprotection, but so far only a few studies have reported a role for amifostine in combinations of radio- and chemotherapy. *In vivo* and clinical data for the use of amifostine in combination with gemcitabine and radiotherapy are still preliminary. In mice, amifostine protected the gemcitabine-radiosensitised mucosa from acute radiation damage [36]. Yavuz and colleagues investigated the potential role of amifostine in combined-modality schedules incorporating gemcitabine and radiotherapy in 10 patients [37]. Based on the higher maximum tolerated dose, they concluded that amifostine might indeed optimise the therapeutic index of gemcitabine-based chemoradiation regimens. Another study is still ongoing [38].

In our study, we investigated the combination gemcitabine and radiation and the cytoprotective agent amifostine *in vitro*. Amifostine with AP clearly showed a protective effect in cells treated with gemcitabine and radiation treatment. In combination with gemcitabine concentrations resulting in a clear radiosensitising effect (4 nM in H292 and 2 nM in ECV304), the protective effect of amifostine and AP was to the same extent as the radioenhancement of gemcitabine. This would mean that amifostine with AP actually negates the radiosensitising

effect of gemcitabine. With a higher concentration of gemcitabine, the protective effect of amifostine with AP was comparable to the effect seen with a lower dose of gemcitabine, which means that the radiosensitising effect of gemcitabine was not completely reduced by amifostine. Protection against the gemcitabine/radiation-induced toxicity is most probably not a direct reduction of the radiosensitisation, but only a radioprotective effect. However, it remains important to know that the protective effect of amifostine in combination with gemcitabine and radiation is to the same extent as the radiosensitising effect of gemcitabine. Thus, if the protection by amifostine *in vivo* selectively occurs in normal tissues, then amifostine should be able to prevent or strongly minimise the increased toxicity that results from the radiosensitising effect of gemcitabine, without influencing the antitumour effect. Therefore, the use of amifostine might be important in the optimisation of this combined therapy.

In conclusion, our *in vitro* model forms a good basis for extensive *in vitro* research on the combination of gemcitabine and radiation treatment. We observed a clear concentration- and cell line-dependent radiosensitising effect of gemcitabine *in vitro*. This study will be expanded with more cell lines and different schedules to further investigate the effects of gemcitabine. Since increased toxicity is an important limiting factor in the combined use of gemcitabine and radiotherapy, our *in vitro* results on the protective effect of amifostine might lead to further improvements in the *in vivo* application of this promising combination.

References

1. Storniolo AM, Allerheiligen SRB, Pearce H. Preclinical, pharmacologic, and phase I studies of gemcitabine. *Semin Oncol* 1997, **24**, 2–7.
2. Peters GJ, Ruiz van Haperen VWT, Bergman AM, *et al*. Preclinical combination therapy with gemcitabine and mechanisms of resistance. *Semin Oncol* 1996, **23**, 16–24.
3. Milas L, Fujii T, Hunter N, *et al*. Enhancement of tumor radioresponse in vivo by gemcitabine. *Cancer Res* 1999, **59**, 107–114.
4. Mason KA, Milas L, Hunter NR, *et al*. Maximizing therapeutic gain with gemcitabine and fractionated radiation. *Int J Radiat Oncol Biol Phys* 1999, **44**, 1125–1135.
5. Shewach DS, Lawrence TS. Gemcitabine and radiosensitization in human tumor cells. *Invest New Drugs* 1996, **14**, 257–263.
6. Chen M, Hough AM, Lawrence TS. The role of p53 in gemcitabine-mediated cytotoxicity and radiosensitization. *Cancer Chemother Pharmacol* 2000, **45**, 369–374.
7. Latz D, Fleckenstein K, Eble M, Blatter J, Wannenmacher M, Weber KJ. Radiosensitizing potential of gemcitabine (2′,2′-difluoro-2′-deoxycytidine) within the cell cycle in vitro. *Int J Radiat Oncol Biol Phys* 1998, **41**, 875–882.
8. Lawrence TS, Chang EY, Hahn TM, Hertel LW, Shewach S. Radiosensitization of pancreatic cancer cells by 2′,2′-difluoro-2′-deoxycytidine. *Int J Radiat Oncol Biol Phys* 1996, **34**, 867–872.
9. Lawrence TS, Davis MA, Hough A, Rehemtulla A. The role of apoptosis in 2′, 2′-difluoro-2′-deoxycytidirne (gemcitabine)-mediated radiosensitization. *Clin Cancer Res* 2001, **7**, 314–319.
10. Mose S, Karapetian M, Juling Pohlit L, *et al*. Radiation enhancement of gemcitabine in two human squamous cell carcinoma cell lines. *Anticancer Res* 2000, **20**(A), 401–405.
11. Ostruszka LJ, Shewach DS. The role of cell cycle progression in radiosensitization by 2′,2′-difluoro-2′-deoxycytidine. *Cancer Res* 2000, **60**, 6080–6088.
12. Rosier JF, Beauduin M, Bruniaux M, *et al*. The effect of 2′-2′ difluorodeoxycytidine (dFdC, gemcitabine) on radiation-induced cell lethality in two human head and neck squamous carcinoma cell lines differing in intrinsic radiosensitivity. *Int J Radiat Biol* 1999, **75**, 245–251.
13. Shewach DS, Hahn TM, Chang E, Hertel LW, Lawrence TS. Metabolism of 2′,2′-difluoro-2′-deoxycytidine and radiation sensitization of human colon carcinoma cells. *Cancer Res* 1994, **54**, 3218–3223.
14. Lawrence TS, Eisbruch A, Shewach DS. Gemcitabine-mediated radiosensitization. *Semin Oncol* 1997, **24**, 24–28.
15. Wachters FM, Kampinga HH, Van Putten JWG, Maring JG, Zdzienicka MZ, Groen HJM. Gemcitabine selectively targets homologous recombination mediated DNA double strand break repair. *Proc Am Ass Cancer Res* 2002, **43**, 740a.
16. Eisbruch A, Shewach DS, Bradford CR, *et al*. Radiation concurrent with gemcitabine for locally advanced head and neck cancer: A phase I trial and intracellular drug incorporation study. *J Clin Oncol* 2001, **19**, 792–799.
17. Ikeda M, Okada S, Tokuuye K, Ueno H, Okusaka T. A phase I trial of weekly gemcitabine and concurrent radiotherapy in patients with locally advanced pancreatic cancer. *Br J Cancer* 2002, **86**, 1551–1554.
18. de Lange SM, van Groeningen CJ, Meijer OWM, *et al*. Gemcitabine-radiotherapy in patients with locally advanced pancreatic cancer. *Eur J Cancer* 2002, **38**, 1212–1217.
19. Mohiuddin M, Kudrimou M, Regine WF, McGrath PC, Hanna N, John W. Concurrent infusional gemcitabine and radiation in the treatment of advanced unresectable GI malignancy: a phase I study. *Cancer J* 2002, **8**, 255–262.
20. McGinn CJ, Zalupski MM, Shureiqi I, *et al*. Phase I trial of radiation dose escalation with concurrent weekly full-dose gemcitabine in patients with advanced pancreatic cancer. *J Clin Oncol* 2001, **19**, 4202–4208.
21. Crane CH, Wolff RA, Abbruzzese JL, *et al*. Combining gemcitabine with radiation in pancreatic cancer: understanding important variables influencing the therapeutic index. *Semin Oncol* 2001, **28**(Suppl. 10), 25–33.
22. Pipas JM, Mitchell SE, Barth RJ, *et al*. Phase I study of twice-weekly gemcitabine and concomitant external-beam radiotherapy in patients with adenocarcinoma of the pancreas. *Int J Radiat Oncol Biol Phys* 2001, **50**, 1317–1322.
23. Trodella L, GrAnone P, Valente S, *et al*. Phase I trial of weekly gemcitabine and concurrent radiotherapy in patients with inoperable non-small-cell lung cancer. *J Clin Oncol* 2002, **20**, 804–810.
24. Culy CR, Spencer CM. Amifostine. An update on its clinical status as a cytoprotectant in patients with cancer receiving chemotherapy or radiotherapy and its potential therapeutic application in myelodysplastic syndrome. *Drugs* 2001, **61**, 641–684.
25. Calabro-Jones PM, Fahey RC, Smoluk GD, Ward JF. Alkaline phosphatase promotes radioprotection and accumulation of WR-1065 in V79-171 cells incubated in medium containing WR-2721. *Int J Radiat Biol* 1985, **47**, 23–27.
26. Smoluk GD, Fahey RC, Calabro-Jones PM, Aguilera JA, Ward JF. Radioprotection of cells in culture by WR-2721 and derivatives:form of the drug responsible for protection. *Cancer Res* 1988, **48**, 3641–3647.
27. Yuhas JM, Spellman JM, Culo F. The role of WR-2721 in radiotherapy and/or chemotherapy. *Cancer Clin Trials* 1980, **3**, 211–216.

28. Brown DQ, Graham WJ, Mackenzie LJ, Pittock JW, Shaw LM. Can WR-2721 be improved upon? *Pharmac Ther* 1988, **39**, 157–168.
29. Calabro-Jones PM, Aguilera JA, Ward JF, Smoluk G, Fahey RC. Uptake of WR-2721 derivatives by cells in culture: identification of the transported form of the drug. *Cancer Res* 1988, **48**, 3634–3640.
30. Capizzi RL. The preclinical basis for broad-spectrum selective cytoprotection of normal tissues from cytotoxic therapies by amifostine. *Semin Oncol* 1999, **26**, 3–21.
31. Pauwels B, Korst AEC, De Pooter CMJ, *et al*. Comparison of the sulforhodamine B test and the clonogenic assay for in vitro chemoradiation studies. *Cancer Chemother. Pharmacol*, in press.
32. Skehan P, Storeng R, Scudiero D, *et al*. New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst* 1990, **82**, 1107–1112.
33. Papazisis KT, Geromichalos GD, Dimitriadis KA, Kortsaris AH. Optimization of the sulforhodamine B colorimetric assay. *J Immunol Methods* 1997, **208**, 151–158.
34. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul* 1984, **22**, 27–55.
35. Leonard CE, Chan DC, Chou TC, Kumar R, Bunn PA. Paclitaxel enhances in vitro radiosensitivity of squamous carcinoma cell lines of the head and neck. *Cancer Res* 1996, **56**, 5198–5204.
36. Phan TP, Crane CH, Janjan NA, Vrdoljak E, Milas L, Mason KA. WR-2721 reduces intestinal toxicity from concurrent gemcitabine and radiation treatment. *Int J Pancreatology* 2001, **29**, 19–23.
37. Yavuz AA, Aydin F, Yavuz MN, *et al*. Radiation therapy and concurrent fixed dose amifostine with escalating doses of twice-weekly gemcitabine advanced pancreatic cancer. *Int J Radiat Oncol Biol Phys* 2001, **51**, 974–981.
38. Jaremtchuk V, Zarba JJ, Keropian M, *et al*. Phase I study of gemcitabine combined with radiotherapy with or without amifostine in patients with locally advanced head and neck cancer. *Proc Am Soc Clin Oncol* 2000, **19**, 424a.