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ORIGINAL ARTICLE

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Chloroacetaldehyde: mode of antitumor action of the ifosfamide metabolite

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Abstract *Background*: The ifosfamide metabolite chloroacetaldehyde had been made responsible for side effects only. We found in previous studies a strong cytotoxicity on human MX-1 tumor cells and xenografts in nude mice. Chloroacetaldehyde is supposed to act via alkylation or by inhibition of mitochondrial oxidative phosphorylation with decrease of ATP. The aim of this study was to further elucidate chloroacetaldehyde's mode of action. *Methods*: MX-1 breast carcinoma cells were measured for ATP-content after exposure to chloroacetaldehyde. Further, the effect of chloroacetaldehyde on DNA-synthesis and its potency of causing strand-breaks or cross-links were investigated by bromodeoxyuridine-incorporation, comet-assay and a DNA interstrand cross-linking-assay. *Results*: Chloroacetaldehyde in high concentrations induces a reduction of ATP-levels when anaerobic glycolysis is blocked by oxamate and reduces the bromodeoxyuridine-incorporation to 46.3% after 4 h when used in IC_{50} concentrations (7.49 μmol/l). In addition we observed DNA single strand-breaks in MX-1 cells treated with chloroacetaldehyde visible in the Comet assay, but no DNA-cross-linking by comet assay and cross-linking assay. *Conclusion*: In summary, our results show that chloroacetaldehyde influences the oxidative phosphorylation in mitochondria, however, this is observed only in high concentrations and is not of clinical relevance because the tumor cells regenerate ATP by anaerobic glycolysis. Nevertheless, chloroacetaldehyde causes DNA-strand-breaks and strong inhibition of DNA-synthesis.

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Keywords Chloroacetaldehyde · Ifosfamide · DNA-synthesis · ATP-content · Single-strand-breaks · DNA–DNA-cross-links

Abbreviations BrdU: Bromodeoxyuridine · ELISA: Enzyme-linked immuno sorbent assay · IC_{50}: Inhibitor concentration 50 · MMS: Methylmethanosulfonate · MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide · PBS: Phosphate-buffered saline · Tris: 2-Amino-2-(hydroxymethyl)-propan-1,3-diol

Introduction

Ifosfamide is a cytostatic drug belonging to the group of oxazaphosphorines and is structurally analogous to cyclophosphamide. The only difference between ifosfamide and cyclophosphamide is a shift of one chlorethylgroup from the exocyclic nitrogen to the nitrogen of the oxazaphosphorine ring causing distinctive drug metabolism [1, 2, 3]. Both alkylating agents are prodrugs requiring cytochrome P450 activation to the 4-hydroxy-metabolite to achieve cancerotoxic activities. In contrast to cyclophosphamide, a second metabolic pathway with considerable liberation of chloroacetaldehyde is important for ifosfamide (Fig. 1). This pathway via N-dechloroethylation is catalyzed by 3A4 and 2B6 enzymes [4]. Side effects such as neuro- [5] and nephrotoxicity [6, 7] but not antitumor activity have been reported for chloroacetaldehyde. We described cytotoxic activity of chloroacetaldehyde in vitro against human solid tumor (MX-1 and S117) and hematologic cell lines (HL-60, THP-1 and HS-Sultan) [8, 9]. This observation could be confirmed in vivo on human xenotransplants in nude mice [10]. However, a contribution of chloroacetaldehyde to the antitumor activity of ifosfamide is not generally accepted [11]. Chloroacetaldehyde might be

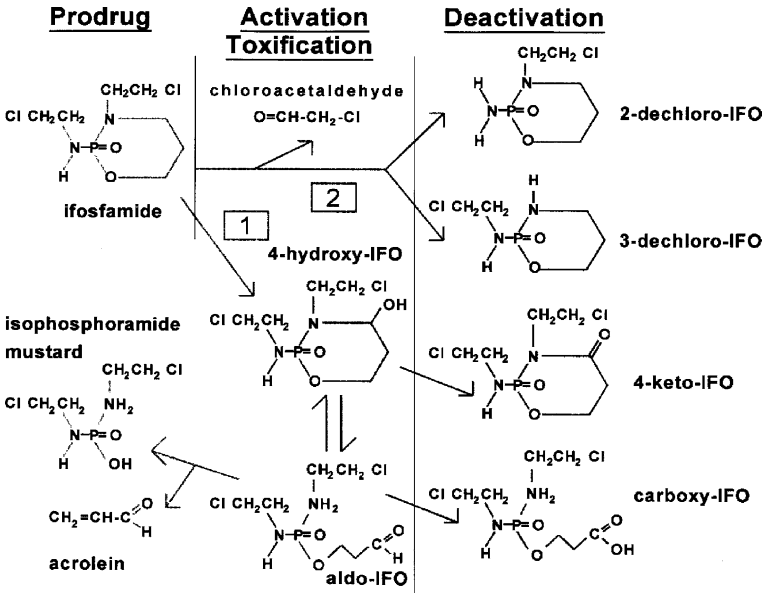

Fig. 1 Metabolism of ifosfamide

responsible for greater antitumor activities of ifosfamide compared to cyclophosphamide in some experimental [12] and human malignancies [13, 14]. It might also explain the lack of complete cross-resistance between the two drugs, which results in activity of ifosfamide even in cyclophosphamide-pretreated patients [14, 15].

Chloroacetaldehyde's mode of action is unclear so far. Chloroacetaldehyde had been proposed to act as an alkylating agent also producing interstrand cross-links [16] and by inhibiting DNA-synthesis [17]. Another possible mode of action is through inhibition of mitochondrial respiration with ATP-depletion [18, 19] and induction of damaging lipid peroxidation leading to cell death [18]. The aim of this study was to elucidate the mode of action of chloroacetaldehyde. The experiments were performed using the MX-1 breast carcinoma cell line. ATP-content after exposure of the cells to chloroacetaldehyde was measured, and the effect of chloroacetaldehyde on DNA-synthesis and its potency of causing cross-links or strand-breaks were investigated by BrdU-incorporation, comet-assay and cross-linking assay. We demonstrate that chloroacetaldehyde at a concentration range observed in human pharmacokinetic studies [9, 20, 21] influences the oxidative phosphorylation in mitochondria and causes strand-breaks and strong and significant inhibition of DNA-synthesis in MX-1 cells.

Methods

Cell lines and cultures

MX-1 breast carcinoma cells (Deutsches Krebsforschungszentrum) were cultured as subconfluent monolayers at 37°C in a humidified atmosphere with 5% CO_2. 80 cm^2 culture flasks (Nunclon) and RPMI 1640 supplemented with 10% fetal calf serum (Biochrom KG), 1% L-glutamine (Roche) and 0.2% penicillin-streptomycin (Life-technologies) were used. The cells were supplied with fresh medium or subcultured twice each week [22].

Reagents

4-Hydroperoxy-ifosfamide and mafosfamide were kindly provided by Baxter Oncology. After dissolution in medium, 4-hydroperoxy-ifosfamide decomposes rapidly to 4-OH-ifosfamide and mafosfamide to equimolar 4-hydroxy-cyclophosphamide and mesna, respectively. Chloroacetaldehyde (Aldrich) was dissolved in normal saline before use and is stable for more than 20 h at room temperature (own data, not published). The addition of equimolar mesna to 4-OH-ifosfamide or chloroacetaldehyde does not change the cytotoxic effects of the two ifosfamide metabolites (own data, not published). Cisplatin (Bristol-Myers Squibb GmbH) and MMS (Merck) were dissolved in medium. The tetrazolium salt MTT (Sigma Chemical Co.) was dissolved in sterile water to 2 mg/ml, sterile filtered and stored in 10 ml aliquots at −20°C until use. Dimethylsulfoxide was obtained from Merck.

Oligomycin A (Sigma-Aldrich Chemical Co.) was dissolved in dimethylsufoxide and diluted with sterile water to the concentrations used in the experiments, oxamate (Sigma-Aldrich) was dissolved in sterile water.

BrdU-assay

The effects on DNA-synthesis were analyzed by BrdU incorporation (Cell Proliferation ELISA, BrdU (colorimetric); Roche). Experiments were performed with 4-hydroperoxy-ifosfamide, chloroacetaldehyde and mafosfamide in IC_{50}. At least ten experiments were carried out, each representing the average of three wells.

One hundred microliter of a 5×10^4/ml cell suspension of MX-1 cells were seeded in a well of a 96 well flat bottom microtiter plate and incubated at 37°C. After 24 h 100 µl of a drug solution were added and the cells were incubated for another 1, 2, 3 or 4 h, respectively. BrdU labeling solution (20 µl) was added in the last hour of incubation. The supernatant was aspirated after the incubation period. The plates were centrifuged for 10 min at 300 *g*, the supernatant was aspirated and the cells were fixed by air drying for 15 min. After the incubation period BrdU assay was performed as follows [23]: 200 µl FixDenat solution (Roche) were added to each well for the fixation of the cells and denaturation of the genomic DNA, exposing the incorporated BrdU to immunodetection. After 30 min incubation at room temperature, 100 µl anti-BrdU-POD were added to locate the BrdU label in the DNA by incubation for another 90 min. The antibody conjugate was removed and the wells were washed three times. The bound anti-BrdU-POD was quantified with a peroxidase substrate (tetramethylbenzidine). Absorbance was measured at 450 nm by using an ELISA plate reader within 5 min after stopping the substrate reaction by adding 25 µl sulfuric acid.

ATP-content

The ATP-content can be measured with a bioluminescence assay [24, 25], we used the ATP Bioluminescence assay Kit CLS II (Roche). One hundred microliter of a 1×10^5/ml cell suspension were seeded into a well of a 96 well flat bottom microtiter plate and incubated at 37°C. After 24 h 20 µl of a drug solution (chloroacetaldehyde, oligomycin, oxamate) were added and the plates were incubated for another 60 min. ATP was extracted with trichloroacetic acid as follows: 100 µl of a 10% trichloroacetic acid were added and the samples were neutralized with 50 µl of a solution of 2 M KOH and Tris buffer (pH 8.3). One hundred microliter of this sample were added to 400 µl Tris/EDTA, centrifuged and 300 µl of the supernatant was filled in tubes for ATP-measurement. The ATP-values were calculated from the measured relative light units. The calibration curves were linear in a range up to 0.6 nmol ATP.

MTT-assay

The MTT-assay [26] carried out after 4 days of cell culture reflects the cell survival. The IC_{50} of the cytotoxic metabolites chloroacetaldehyde, 4-OH-ifosfamide and mafosfamide was determined by using the MTT-assay performed with minor modifications [22]. MX-1 cells were treated with 100 µl drug solution 24 h after seeding 100 µl of a 5×10^4/ml cell suspension in wells of microtiter plates. MTT (25 µl) was added after 4 days of incubation at 37°C and the MTT assay was carried out as described previously [22].

Comet assay

DNA strand-breaks or cross-links were detected with an alkaline version [27] of the Comet assay [28]. MX-1 cells were treated with 1 ml drug solution 24 h after seeding 2×10^6 cells in a 25 cm^2 culture flask. The cells were incubated for 4 h at 37°C, quickly transferred into a 50-ml tube after trypsination, washed and resuspended in 50 µl PBS. Frosted glass slides were each covered with 250 µl of 0.75% normal melting point agarose in PBS at 60°C, then carefully covered with a coverslip and kept at 4°C for 15 min to allow the agarose to solidify. This first layer is used to promote even and firm attachment of the second and third layer. Treated or control cells (150,000 each) suspended in 50 µl of PBS were mixed with 333 µl of 0.5% low melting point agarose at 37°C. After gently removing the coverslip 75 µl of the cell suspension were pipetted onto the first layer of agarose. For spreading cells evenly, the slide has to be covered with a coverslip again and maintained at 4°C for 15 min. The third layer is added after removal of the coverslip only for the protection of the cells. It consists of 100 µl of 0.5% low melting point agarose applied at 37 °C which is spread using a coverslip and solidify at 4°C for another 15 min. Following slide preparation, the embedded cells were lysed by gently immersing the slides in a freshly prepared cold lysis buffer at alkaline pH (11.5) for 1 h (2.5 M NaCl, 100 mmol/l EDTA, 10 mmol/l Tris, 1% sodium sarcosinate, with 1% Triton X and 10% dimethylsulfoxide added just before use). To allow unwinding of the DNA, the slides were equilibrated in cold alkaline electrophoresis solution (300 mmol/l NaOH and 1 mmol/l EDTA, pH 11) for 20 min prior to electrophoresis. Electrophoresis was conducted for 25 min at 25 V, adjusted to 300 mA by raising or lowering the buffer level in the tank. Following electrophoresis, slides were washed with PBS three times for 5 min and stained with 40 µl of ethidium bromide (20 µg/ml). Images were visualized immediately with the use of a ZEISS fluorescence microscope with a CY-3 filter.

DNA isolation and fluorometric assay for the determination of DNA–DNA interstrand cross-links

The fluorometric assay is based on the ability of blue fluorescent Hoechst 33258 to bind double strand DNA [29]. Calf thymus DNA and DNA isolated from treated MX-1 cells were used for the experiments. The isolation of human genomic DNA from MX-1 cells was performed according to a modified version of the Nucleon protocol from Scotlab GmbH [30]. This method gives a high yield and purity of DNA with high molecular weight. After trypsination, the cells were washed and resuspended with 2 ml of a solution containing 400 mmol/l Tris–HCl, 60 mmol/l EDTA, 150 mmol/l NaCl, 1% sodium dodecylsulfate. Five hundred micro-

liter sodium perchlorate were added, the suspension was mixed at room temperature for 15 min and incubated in a shaking water bath for 25 min at 65°C followed by adding 2 ml of −20°C chloroform. The tubes were mixed for 10 min at room temperature, centrifuged and the aqueous DNA-containing layer was transferred into a 50-ml tube with 5 ml of 4°C ethanol (100%). The precipitated DNA was transferred into a 1.5-ml tube, washed twice with 1 ml 4°C ethanol (70%), dried at room temperature and dissolved in 50 µl TE buffer (10 mmol/l Tris–HCl, 1 mmol/l EDTA). Calf thymus DNA (125 µg/ml) dissolved in TE buffer also was exposed to the cross-linking agent cisplatin and to chloroacetaldehyde at 37°C for 2 and 4 h or kept at 37°C without additives (control sample). After DNA isolation, aliquots (15 µl) were transferred into a glass test tube containing 0.1 µg/ml Hoechst 33258 in 1.5 ml of 5 mmol/l Tris-HCl, 0.5 mmol/l EDTA buffer (pH 8.0), yielding a final DNA concentration of 1.25 µg/ml. Fluorescence was recorded using a Spectrofluorophotometer RF1501 (Shimadzu) (excitation 365 nm, emission 460 nm). The samples were heated to 96°C for 5 min and immediately submerged into an ice-water-bath. After incubation for 5 min, the samples were transferred into a water bath at room temperature for additional 5 min and the fluorescence was recorded again. To calculate the proportion of DNA specifically cross-linked in response to treatment, referred to as "cross-linked fraction," the obtained fluorescence values were calculated as follows:

$$\text{Cross-linked fraction} = \frac{E_A/E_B - C_A/C_B}{1 - C_A/C_B}$$

E_A fluorescence of the experimental sample after heat/chill
E_B fluorescence of the experimental sample before heat/chill
C_A fluorescence of the control sample after heat/chill
C_B fluorescence of the control sample before heat/chill

Results

BrdU-assay

The influence of chloroacetaldehyde and 4-OH-ifosfamide in comparison to the active cyclophosphamide metabolite mafosfamide on DNA-synthesis was tested in IC_{50} which was determined by using the MTT-assay as described in the methods (see also legend to Fig. 2). The IC_{50s} determined in vitro are in the same range of plasma concentrations measured in human pharmacokinetics [9]. An incubation of MX-1 cells with chloroacetaldehyde in clinically relevant concentrations [9] for one to 4 h leads to a time-dependent reduction of BrdU-incorporation (Fig. 2). BrdU-incorporation is decreased to 46.9% after 4 h of incubation with chloroacetaldehyde ($P < 0.01$) which is in the same range of 4-OH-ifosfamide's and mafosfamide's effect.

ATP-content

Table 1 shows the effect of chloroacetaldehyde on ATP levels of MX-1 tumor cells. Treatment of the cells with chloroacetaldehyde showed a concentration-dependent reduction of ATP-levels. However, in concentrations up to 100 µmol/l, this effect was only seen when anaerobic glycolysis was blocked by oxamate [31, 32]. Experiments without oxamate did not show diminished ATP-levels of MX-1 cells after incubation with chloroacetaldehyde, suggesting that the cells might regenerate ATP via anaerobic glycolysis. In fact, also the effect of oligomycin, which is known to blocking aerobic glycolysis [33] could only be seen, when anaerobic glycolysis was blocked by oxamate. The ATP-level after 60 min incubation of MX-1 with oligomycin (50 µmol/l) was 93.18%, after incubation with oligomycin (10 µmol/l) in combination with oxamate (100 µmol/l) was 1.64%.

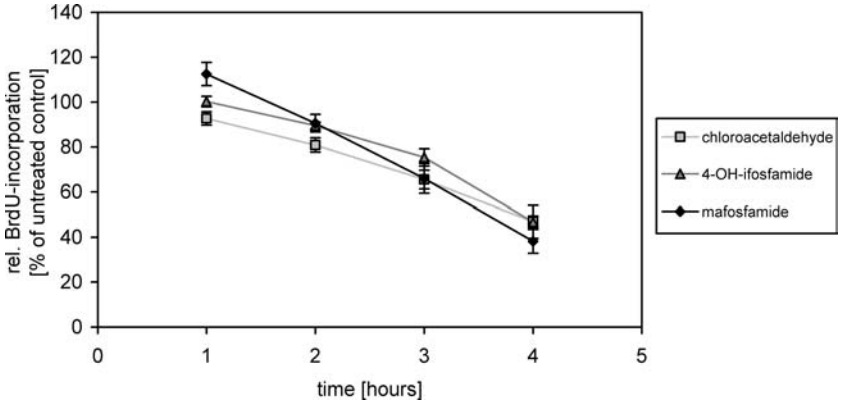

Fig. 2 Relative BrdU-incorporation of MX-1 tumor cells after 1–4 h incubation with chloroacetaldehyde ($n = 13$), 4-hydroxy-ifosfamide ($n = 12$) or mafosfamide ($n = 12$) in IC_{50}(chloroacetaldehyde 7.49 µmol/l, 4-OH-ifosfamide 15 µmol/l, mafosfamide 8.61 µmol/l); Mean ± SEM; *$P < 0.01$

Table 1 Influence of chloroacetaldehyde, oligomycin, oxamate and a combination of oxamate with oligomycin or chloroacetaldehyde on ATP-levels of MX-1 tumor cells after 60 and 120 min incubation, $n = 6$

	ATP (nmol/10^6 cells) ± SEM (percentage of untreated control)	
	60 min	120 min
Control	1.60 ± 0.05 (100)	1.72 ± 0.04 (100)
Chloroacetaldehyde		
20 μM	1.59 ± 0.04 (99.1)	1.53 ± 0.03 (88.6)
100 μM	1.52 ± 0.01 (94.9)	1.50 ± 0.03 (86.9)
500 μM	1.47 ± 0.01 (91.5)	0.058 ± 0.001 (33.5)
Control	1.07 ± 0.03 (100)	1.14 ± 0.02 (100)
Oxamate 100 μM + oligomycin 10 μM	0.02 ± 0.001 (1.64)	0.01 ± 0.00 (0.96)
Control	1.14 ± 0.02 (100)	1.17 ± 0.02 (100)
Oxamate 100 μM + chloroacetaldehyde	0.37 ± 0.004 (31.3)	0.34 ± 0.004 (28.3)
20 μM	0.34 ± 0.003 (28.9)	0.28 ± 0.003 (24.6)*
100 μM	0.31 ± 0.016 (27.8)	0.25 ± 0.002 (22.1)**
500 μM	0.13 ± 0.001 (11.4)**	0.03 ± 0.00 (2.1)**

*$P < 0.05$; **$P < 0.01$

Comet-assay

Alkylating properties of chloroacetaldehyde were investigated by using the comet-assay [34, 35] in comparison to the known strand-breaking agent MMS and to the cross-linking drug cisplatin. The electrophoretic mobility of cross-linked DNA strands is reduced when compared with single strand breaks. As a consequence, comet tails of cells treated with MMS and cisplatin are shorter than those of cells treated with MMS alone. Chloroacetaldehyde in clinically relevant concentrations caused comet tails of the MX-1 cells with a high density and length similar to those of MMS-treated cells. This effect was dose dependent (Fig. 3). A combination treatment with MMS and chloroacetaldehyde enhanced the density and length of comet tails as compared to MMS-only treated cells. As the tail length was not reduced this suggests that chloroacetaldehyde causes DNA single-strand-breaks but not cross-links.

Cross-linking assay

The cross-linking fraction of cisplatin in a concentration of 100 μmol/l reached up to 13.7% after 2 h and up to 37.8% after 4 h incubation. In contrast, the DNA–DNA cross-linking fraction for chloroacetaldehyde (20–80 μmol/l and 800 μmol/l) was below the limit of detection when tested with MX-1 cells. The results with calf thymus DNA are similar. Again, the cross-linking fraction for chloroacetaldehyde (20–80 μmol/l and 8,000 μmol/l) was below the limit of detection.

Discussion

Chloroacetaldehyde is the metabolite of side chain oxidation of ifosfamide made responsible for side effects only. We were the first who showed a strong in vitro cytotoxicity of chloroacetaldehyde against human tumor cells in a drug concentration range equivalent to plasma concentrations measured in patients treated with 5 g/m^2 ifosfamide [9]. In addition, antitumor activity of chloroacetaldehyde could be confirmed by us in vivo in human tumor xenografts [10]. With respect to the area under the curve of chloroacetaldehyde and 4-OH-ifosfamide measured in the blood, the effect of chloroacetaldehyde was at least as high as for 4-OH-ifosfamide. In order to elucidate the mode of action of chloroacetaldehyde, we investigated its effect on DNA-synthesis, oxidative phosphorylation and its potency of causing DNA strand breaks or cross links. We found that chloroacetaldehyde diminishes ATP-levels by blocking the oxidative phosphorylation in mitochondria. However, this effect is not of clinical relevance, because it is observed only in high concentrations of chloroacetaldehyde and the tumor cells regenerate ATP via anaerobic glycolysis. We also found that chloroacetaldehyde causes DNA strand breaks and strong inhibition of DNA synthesis.

The decrease of BrdU-incorporation in concentrations equivalent to those measured in human pharmacokinetics [9] shows that chloroacetaldehyde causes early inhibition of DNA-synthesis. Our findings are in good accordance with the results of Kandala et al. [17], who observed a significant decrease of DNA-synthesis NIH-3T3 cells after treatment with chloroacetaldehyde. As demonstrated by our results an inhibition of oxidative phosphorylation with consecutive ATP loss as described by Sood and O'Brien [18] or the induction of cross-links as described by Spengler and Singer [16] seem not to be an explanation for the impaired DNA-synthesis.

Sood and O'Brien [18] showed a 50% decrease of hepatocyte viability after incubation with high concentrations of 300 μmol/l chloroacetaldehyde for 2 h [18] and found inhibition of hepatocyte respiration and ATP depletion to be the earliest cytotoxic effects of chloroacetaldehyde responsible for the reduction of cell viability [18]. They reported an inhibition of hepatocyte respiration which was time dependent as shown by a decline of O_2 uptake of hepatocytes after 60 min

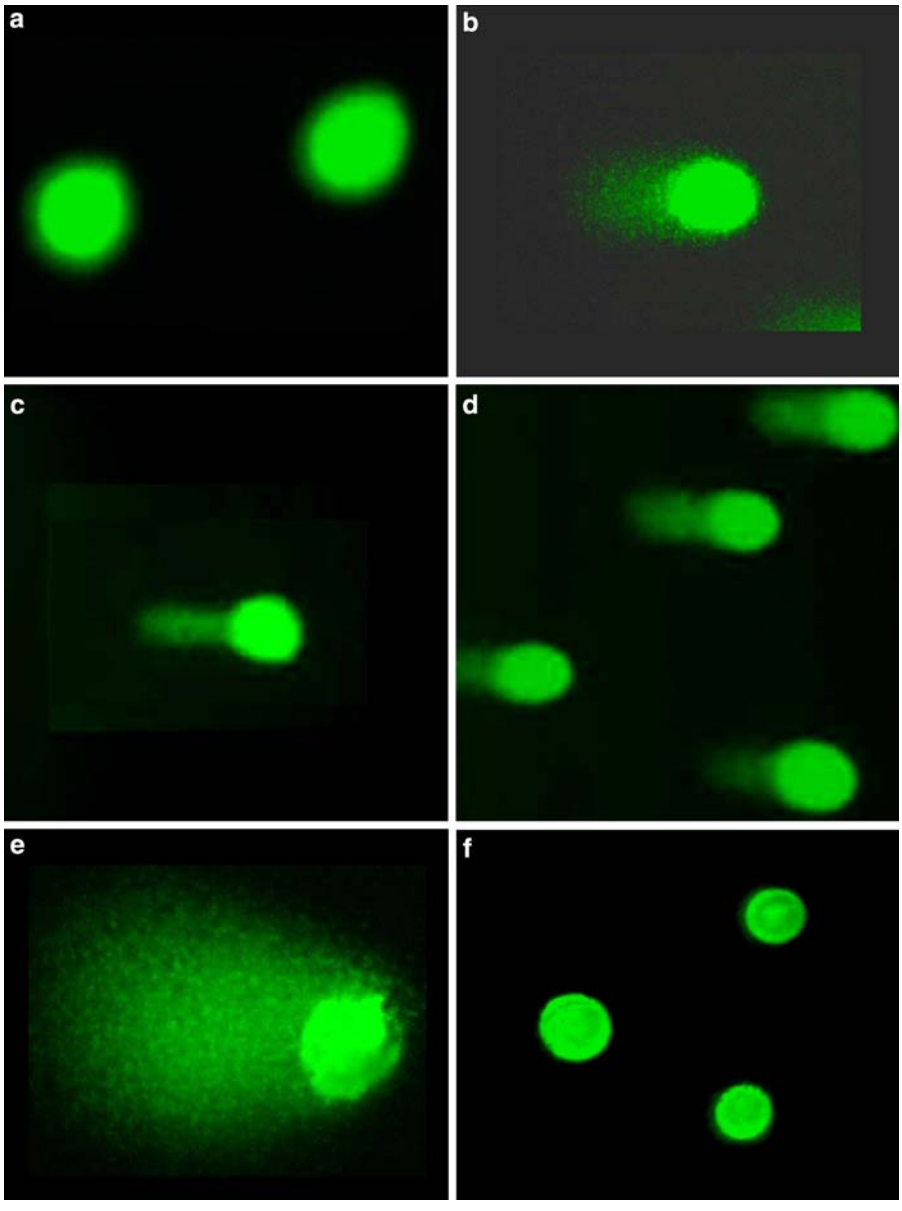

Fig. 3 Comet assay after 4 h incubation of MX-1cells with chloroacetaldehyde, MMS, cisplatin (1:400). **a** untreated control, **b** MMS 100 µmol/l, **c** chloroacetaldehyde 20 µmol/l, **d** chloroacetaldehyde 80 µmol/l, **e** MMS 100 µmol/l + chloroacetaldehyde 80 µmol/l, **f** MMS100 µmol/l + cisplatin 100 µmol/l

incubation with 500 µmol/l chloroacetaldehyde. As a consequence, also the reduction of hepatocyte ATP levels was shown to be time dependent with a loss of more than 50% after 30 min incubation with 500 µmol/l chloroacetaldehyde. The reason for those high concentrations of chloroacetaldehyde needed to decrease viability, oxidative phosphorylation and ATP-content in hepatocytes may be that hepatocytes contain high amounts of aldehydedehydrogenases, which may inactivate chloroacetaldehyde. On the other hand, as with acetaldehyde, aldehydedehydrogenases should oxidize chloroacetaldehyde to chloroacetic acid, which does not imply deactivation. The formation of chloroacetyl-CoA from chloroacetic acid may disturb CoA-dependent mechanisms by 'lethal synthesis' [36] as it was also described for fluoroacetic acid [37]. Therefore, despite the known inactivation of aldoifosfamide to carboxyifosfamide by aldehdedehydrogenases in ifosfamide metabolism (Fig. 1) [3], the influence of aldehydedehydrogenases on chloroacetaldehyde-metabolism remains unclear. In addition to the results of Sood and O'Brien, Visarius et al. [38] found a 4-fold decrease in oxidative metabolism of 500 µmol/l chloroacetaldehyde in rat liver mitochondria and demonstrated that chloroacetaldehyde inhibits the oxidation of long-chain fatty acids in rats [39].

Like Sood and O'Brien [18], we found a reduction of ATP in MX-1 tumor cells after chloroacetaldehyde exposure. However, chloroacetaldehyde's potential to reduce ATP by inhibiting the oxidative phosphorylation could only be shown when the anaerobic glycolysis of the tumor cells was blocked by oxamate and when chloroacetaldehyde was used in very high and clinically irrelevant concentrations of 500 µmol/l, suggesting that the cells could regenerate ATP via anaerobic glycolysis. In fact, the ATP-generation via

oxidative phosphorylation is low for many tumors cells because of a defective mitochondrial system [40]. Hypoxic tumor cells generate ATP via anaerobic glycolysis. Rossignol et al. [40] showed that HeLa cells can adapt their mitochondrial network structurally and functionally to derive energy by glutaminolysis only. In addition, a decreased capacity of oxidative phosphorylation is supposed to be responsible for faster tumor growth or increased invasiveness [41]. Taken together, the inhibition of the oxidative phosphorylation by chloroacetaldehyde in a concentration range of 50–70-fold above clinical concentrations is not relevant.

Our experiments with pure DNA or DNA isolated from MX-1 cells after incubation with chloroacetaldehyde or cisplatin as a positive control showed no cross-linking activity of chloroacetaldehyde even in very high concentrations of 8,000 μmol/l. The finding of Spengler and Singer [16] who demonstrated cross-linking by chloroacetaldehyde, may be explained by the use of very high concentrations of up to 500,000 μmol/l chloroacetaldehyde. This concentration of chloroacetaldehyde is more than 10,000-fold higher than the concentrations of chloroacetaldehyde observed in human pharmacokinetics. In contrast to the lack of cross-linking activity in the presented experiments, chloroacetaldehyde was found to produce single-strand-breaks in MX-1 tumor cells in a concentration dependent manner. Although the comet assay is not very sensitive, the microscopic detection limit for strand-breaks following chloroacetaldehyde incubation was at IC_{50}. Optimal strand-breaking with good visible comet-like figures as shown in Fig. 3 was documented when concentrations of 1.5-fold above clinical concentrations were used (20 μmol/l). Kuchenmeister et al. [42] found strand-breaks caused by chloroacetaldehyde, but they used up to 1,000-fold higher concentrations for their experiments with rat hepatocytes (1.1 mmol/l; 2.9 mmol/l; 11.5 mmol/l).

In summary, chloroacetaldehyde influences the oxidative phosphorylation of mitochondria. However, this is not of clinical relevance, because the tumor cells regenerate ATP via anaerobic glycolysis. Nevertheless, chloroacetaldehyde in concentrations measured in human pharmacokinetics [9] acts as an alkylating agent inhibiting DNA-synthesis through DNA strand-breaking effects without cross-linking activity. The generation of chloroacetaldehyde in the metabolism of ifosfamide may explain the differing antitumoral activities of ifosfamide and cyclophosphamide and also the lack of complete cross-resistance beween these drugs [13, 14]. Although chloroacetaldehyde is generated in the presence of other toxic metabolites like isophosphoramide mustard and acrolein, it contributes to the cytotoxic activity of ifosfamide [9, 10]. Thus, suppression of chloroacetaldehyde metabolic pathway to avoid neurotoxic side effects as proposed by Brain et al. [43] may also be associated with a reduction of antitumor effects.

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