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Cisplatin combined with Zidovudine enhances cytotoxicity and oxidative stress in human head and neck cancer cells via a thiol dependent mechanism

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Abstract

Oxidative stress and mitochondrial dysfunction in cancer cells represent features that may be exploited therapeutically. We determined if agents which induce mitochondrial dysfunction such as zidovudine (AZT) and cisplatin (CIS) could enhance killing of human head and neck cancer cells via oxidative stress. AZT and/or CIS-induced cytotoxicity was determined using clonogenic survival, mitochondrial membrane potential was analyzed to investigate mitochondrial function, and glutathione was measured to determine thiol metabolism perturbations. AZT+CIS significantly increased toxicity and reduced mitochondrial membrane potential in FaDu, Cal-27 and SQ20B head and neck cancer cells while increasing % glutathione disulfide (GSSG). Treatment with the thiol antioxidant, N-acetylcysteine (NAC), reversed the loss of mitochondrial membrane potential and the increase in %GSSG, and partially protected FaDu and Cal-27 cells from AZT+CIS. Finally, an inhibitor of glutathione synthesis, L-buthionine-[S,R]-sulfoximine, sensitized the cells to AZT+CIS-induced cytotoxicity which was partially reversed by NAC. These results suggest that exposure of cancer cells to agents which induce mitochondrial dysfunction such as AZT cause significant sensitization to CIS-induced toxicity via disruptions in thiol metabolism and oxidative stress. These findings provide a biochemical rationale for evaluating agents which induce mitochondrial dysfunction in combination with chemotherapy and inhibitors of glutathione metabolism in head and neck cancer.

Keywords

cisplatin; azidothymidine; cancer; oxidative stress; mitochondrial membrane potential; NAC; BSO

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Introduction

For decades, reactive oxygen species (ROS) production and metabolic oxidative stress have been associated with cancer [1-8]. Growing evidence suggests that cancer cells exhibit increased intrinsic metabolic oxidative stress compared to normal untransformed cells [2-5], due in part to mitochondrial dysfunction [5-7] and increased metabolic activity [8]. Since mitochondrial electron transport complexes (ETCs) are a major source of ROS generation in the cells [9], ROS-mediated damage to mitochondrial DNA (mtDNA) may be a mechanism which could amplify metabolic oxidative stress in cancer cells that would promote genetic instability and progression of the malignant phenotype [10,11]. We proposed that if cancer cell mitochondria exhibit increased ROS production from inherent mitochondrial dysfunction, then further mitochondrial injury may selectively increase oxidative stress in cancer cells and enhance susceptibility to conventional chemotherapeutic agents thought to induce oxidative stress.

Cisplatin (cis-diamminedichloroplatinum(II), CIS) and Zidovudine (azidothymidine, AZT) are two commonly used agents that have been shown to induce mitochondrial dysfunction and cytotoxicity in cancer cells (12-16). The purpose of the current studies was to test the hypothesis that AZT in combination with CIS enhances cytotoxicity by mechanisms involving disruptions in mitochondrial and thiol metabolism leading to metabolic oxidative stress in human head and neck cancer cells.

Materials and Methods

Cells and culture conditions

FaDu and Cal-27 human head and neck squamous carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). SQ20B human head and neck squamous carcinoma cells were kindly provided by Dr. Anjali Gupta (Department of Radiation Oncology, University of Iowa, Iowa City, IA). All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4 mM L-glutamine, 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate and 4.5 g/L glucose with 10% fetal bovine serum (FBS; Hyclone, Logan, UT). Cultures were maintained in 5% CO₂ and humidified in a 37°C incubator.

Drug Treatment

Zidovudine (Azidothymidine, AZT), N-acetyl cysteine (NAC) and L-buthionine-[S,R]-sulfoximine (BSO) were obtained from Sigma Chemical Co. (St. Louis, MO). Cis-diamminedichloroplatinum(II) (cisplatin, CIS) was obtained from Bedford Laboratories (Bedford, OH). All drugs were used without further purification. Drugs were added to cells at final concentrations of 10-1000 µM AZT, 0.2 µM CIS, 20 mM NAC and 1.0 mM BSO. All stock solutions were dissolved in PBS except NAC was dissolved in 1 M sodium bicarbonate (pH 7.0), and the required volume added directly to complete cell culture media on cells to achieve the desired final concentrations. All cells were placed in a 37°C incubator and harvested at the time points indicated.

Glutathione assay

Cell pellets were thawed and homogenized in 50 mM PO₄ buffer (pH 7.8) containing 1.34 mmol/L diethylenetriaminepentaacetic acid (DETAPAC) buffer. Total glutathione content was determined by the method of Anderson [17]. Reduced glutathione (GSH) and glutathione disulfide (GSSG) were distinguished by addition of 2 µL of a 1:1 mixture of 2-vinylpyridine and ethanol per 30 µL of sample followed by incubation for 1 h and assayed as described previously [18]. All glutathione determinations were normalized to the protein content of whole homogenates using the method of Lowry et al. [19].

Clonogenic cell survival experiments

Attached cells from experimental dishes were trypsinized with 1 mL trypsin-EDTA (CellGro, Herndon, VA) and inactivated with DMEM containing 10% fetal bovine serum (Hyclone). The cells were diluted and counted using a Coulter counter. Cells were plated at low density (300-1,000 per plate), and clones were allowed to grow in a humidified 5% CO₂, 37°C environment for 14 days in complete medium, in the presence of 0.1% gentamicin. Cells were fixed with 70% ethanol and stained with Coomassie blue for analysis of clonogenic cell survival as previously described [20]. Individual assays were performed with multiple dilutions with at least four cloning dishes per data point, repeated in at least 3 separate experiments.

Mitochondrial membrane potential

Attached cells were incubated with 10 µg/mL JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide; Invitrogen, Eugene, OR) and resuspended in media with or without 50 µM carbonyl cyanide 3-chlorophenylhydrazone (CCCP; Sigma, St. Louis, MO) which was used as a positive control. Cells were incubated at 37°C for 15 min, trypsinized then spun at 500 × g. The pellet was resuspended in 500 µL of PBS. The samples were filtered through 36 micron mesh and analyzed for fluorescence in FL1 and FL2 channels using a Becton Dickinson FACS machine.

Statistical analysis

Statistical analysis was done using GraphPad Prism version 4 for Windows (GraphPad Software, San Diego, CA). To determine differences between 3 or more means, one-way ANOVA with Tukey post-tests were performed. Error bars represent the standard error of the mean. All statistical analysis was performed at the p<0.05 level of significance.

Results

Effect of AZT on FaDu survival

To investigate the effect of AZT on human head and neck squamous carcinoma cell survival, FaDu, Cal-27 and SQ20B cells were treated with increasing doses of AZT (10-1000 µM) for 24 h and analyzed for clonogenic survival. The results show that 10 and 30 µM AZT did not significantly alter cell survival when compared to control in all three cell lines (Fig. 1). However, further increasing the dose of AZT up to 100, 300 and 1000 µM caused significant cell killing compared to control in FaDu cells (Fig. 1). Cal-27 and SQ20B cells were more resistant to AZT treatment compared to FaDu, with significant cell killing observed only at the AZT dose of 1000 µM. These results show that the cytotoxic effect of AZT in head and neck cancer cells is dose dependent and varies by cell type.

Enhancement of CIS-induced cytotoxicity with AZT

We assessed the effects of AZT and CIS as single agents and in combination on clonogenic survival in FaDu, Cal-27 and SQ20B cells. 300 µmol/L AZT was chosen for the current studies because it is well below the maximum plasma concentrations observed (600 µmol/L) after administration of 1.5–7 g/m² AZT over 2 h in patients with advanced cancer (21). In addition, 0.2 µmol/L CIS was chosen because it represents a clinically relevant dose that is within the achievable plasma levels in head and neck patients during CIS therapy (22). Clonogenic assays were performed following treatment with AZT and/or CIS for 24 h. Treatment with AZT caused significant cell killing in FaDu (65 %, p<0.01), Cal-27 (37 %, p<0.05) and in SQ20B (23 %, p<0.05), while CIS only caused significant cell killing in FaDu cells (30 %, p<0.01) compared to control (Fig. 2A). The combination of AZT and CIS caused a significant increase in cell killing compared to AZT or CIS alone in FaDu and Cal-27 to 86 and 57 % cell killing respectively (p<0.05), while SQ20B showed a trend towards increased cell killing that did not

reach significance (39 % cell killing, $p>0.05$, Fig. 2A). These results show at least additive in FaDu and Cal-27 cells or possibly a more than additive effect of AZT and CIS in head and neck cancer cell lines.

AZT and CIS induced loss of mitochondrial membrane potential

To determine if AZT and/or CIS induced mitochondrial dysfunction in FaDu cells, we measured relative mitochondrial membrane potential using JC-1. JC-1 is a cationic dye with a fluorescence in the 535 nm range (FL1 or the green channel) that is used as an indicator of mitochondrial membrane potential in cells. In healthy intact mitochondria, JC-1 is electrostatically attracted into the mitochondria and forms aggregates that fluoresce in the 585 nm range (FL2 or the red channel). However, if the mitochondria are relatively depolarized, less aggregates accumulate in the mitochondria resulting in less red fluorescence. Thus, the ratio of FL2/FL1 fluorescence decreases following membrane depolarization and can be used as an indicator of loss of mitochondrial membrane potential. Treatment with AZT caused a significant decrease in the FL2/FL1 ratio in FaDu and SQ20B while CIS did not significantly alter the FL2/FL1 ratio in any of the cell lines (Fig. 2B). However, AZT+CIS caused a significant decrease in the FL2/FL1 ratio in all three cell lines compared to untreated control cells (Fig. 2B). There was a dramatic decrease in the FL2/FL1 ratio ($< 12\%$ of control treated cells) when the positive control (CCCP) was used in combination with each treatment (data not shown). These results support the hypothesis that AZT and CIS cause loss of mitochondrial membrane potential suggesting disruptions in mitochondrial function.

AZT in combination with CIS induced disruptions in glutathione metabolism consistent with oxidative stress

We examined if oxidative stress could be contributing to the cytotoxicity of AZT and CIS by measuring glutathione (GSH/GSSG) levels in FaDu cells. The GSH/GSSG redox couple represents the major small molecular weight thiol-disulfide redox buffer in cells (23). The amount of total GSH that was oxidized was used to calculate % GSSG. Consequently, an increase in % GSSG is believed to signify a shift towards a more highly oxidizing intracellular environment indicative of oxidative stress (23). AZT and AZT+CIS caused a significant decrease in total GSH levels in SQ20B cells but did not significantly change total GSH levels in FaDu and Cal-27 cells (Fig. 2C). However, a significant increase in % GSSG was observed when the cells were treated with AZT+CIS (Fig. 2D) compared to untreated cells in all three cell lines ($p<0.01$). These results support the hypothesis that the toxicity of AZT in combination with CIS may be in part mediated by disruptions in glutathione and/or thiol metabolism consistent with causing oxidative stress.

Inhibition of AZT and CIS-induced cytotoxicity by the thiol antioxidant, NAC

To analyze the involvement of oxidative stress in AZT and CIS-induced cytotoxicity, FaDu and Cal-27 cells were treated with 20 mM of the thiol antioxidant N-acetyl cysteine (NAC) for 1 h before and during exposure to AZT and CIS, then analyzed for clonogenic survival. SQ20B cells were not analyzed in these experiments because of NAC-induced cytotoxicity in this cell line. NAC partially but significantly rescued the cytotoxicity induced by the combination of AZT and CIS (52%: NAC+AZT+CIS vs. 86%: AZT+CIS, $p<0.01$) in FaDu cells (Fig. 3A). NAC also partially rescued Cal-27 cells from AZT+CIS-induced cytotoxicity but this effect did not reach significance (45%: NAC+AZT+CIS vs. 57% AZT+CIS, $p>0.05$, Fig. 3A). These results show that treatment with a thiol antioxidant is able to partially inhibit the cytotoxicity induced by AZT in combination with CIS in human head and neck cancer cells.

AZT and CIS-induced loss of mitochondrial membrane potential is reversed by NAC

To investigate if NAC was able to inhibit the loss of mitochondrial membrane potential induced by AZT and CIS, FaDu and Cal-27 cells were labeled with JC-1 for 15 min after treatment with AZT in combination with CIS with or without pretreatment with NAC. NAC significantly and completely reversed the loss of mitochondrial membrane potential induced by AZT in combination with CIS in both cell lines (Fig. 3B). These results support the hypothesis that disruptions in thiol metabolism may be responsible for the loss of mitochondrial membrane potential induced by AZT+CIS.

AZT and CIS-induced disruptions in glutathione metabolism are inhibited by NAC

To determine if NAC caused any effects on intracellular GSH/GSSG in FaDu and Cal-27 cells treated with AZT and CIS, cells were harvested for glutathione analysis after treatment with AZT in combination with CIS with or without pretreatment with NAC. Although treatment with AZT+CIS did not alter total GSH levels, cells exposed to NAC alone and NAC in combination with AZT and CIS significantly increased total GSH (Fig. 3C). More importantly, NAC significantly decreased the % GSSG induced by AZT and CIS, which was comparable to NAC alone (Fig. 3D). Taken together, Figure 3 supports the hypothesis that the cytotoxic effects of AZT in combination with CIS are mediated by loss of mitochondrial membrane potential and disruptions in thiol metabolism consistent with oxidative stress, which was reversed by the thiol antioxidant, NAC.

AZT and CIS-induced cytotoxicity is enhanced by BSO

To determine if GSH depletion would further enhance the cytotoxicity induced by AZT and CIS, cells were treated with 1 mM BSO, which is an inhibitor of GSH synthesis, for 1 h before and during treatment with AZT and CIS for 24 h. The results indicate that BSO significantly depleted total GSH to less than 15 % of control cells in FaDu, Cal-27 and SQ20B cells (Fig. 4). Furthermore, the cell killing observed with the combination of AZT and CIS was significantly enhanced by BSO in FaDu and Cal-27 cells, and showed a trend towards enhancement in SQ20B cells (Fig. 4). Finally, NAC also significantly protected against the cytotoxicity of AZT+CIS+BSO in FaDu cells (Fig. 5). These data show that depletion of glutathione (GSH) with BSO enhances the cytotoxicity observed with AZT and CIS and treating with a non-specific thiol antioxidant protected against this toxicity.

Discussion

Mitochondrial dysfunction is believed to cause cellular damage and death by disrupting the mitochondrial membrane potential and increasing ROS production (24,25). If cancer cells are already under metabolic oxidative stress due to dysfunctional mitochondria, then agents capable of inducing further mitochondrial dysfunction such as AZT and CIS would be predicted to enhance cytotoxicity by increasing metabolic oxidative stress. Here we show data consistent with the hypothesis that AZT in combination with CIS causes a loss in mitochondrial membrane potential as well as enhanced cytotoxicity in FaDu, Cal-27 and SQ20B head and neck cancer cells by mechanisms that appear to involve disruptions in thiol metabolism and increases in parameters indicative of oxidative stress.

Cisplatin is one of the most commonly used chemotherapeutic drugs used in the treatment of head and neck cancer (26). Cisplatin-induced cytotoxicity is believed to be caused by the formation of DNA adducts which have been suggested to accumulate preferentially in mtDNA (26,27). Cisplatin has also been associated with the increased production of ROS and biochemical manipulations aimed at reducing ROS production or detoxifying ROS species have resulted in decreased toxicity of cisplatin (13,28). Zidovudine (3'-Azido-3'-deoxythymidine, AZT) is a thymidine nucleoside analog commonly used in the treatment of

HIV (human immunodeficiency virus) infection (29,30). AZT has been shown to block the nucleoside-binding site of the viral reverse transcriptase and to inhibit DNA replication by chain termination *in vitro* (31-33). AZT incorporates into both nDNA and mtDNA but binds preferentially to mtDNA leading to mtDNA depletion and cell death (15,32,33). In addition, AZT has been shown to interfere with regulation of complex 1 and induce $O_2^{\bullet-}$ production (16).

Studies have shown that AZT at doses ranging from 0.1 – 100 μ M inhibited the growth of MCF-7 human breast cancer cells, and the growth of N-methyl-N-nitrosourea (MNU) induced rat mammary tumors (34). AZT also caused approximately 20 % clonogenic cell killing in T47D and MCF-7 cells after treatment with 0.75 and 1.35 mM AZT respectively (35). In our experiments, we have shown that in FaDu, Cal-27 and SQ20B human head and neck cancer cells, AZT at 1 mM for 24 h caused 44, 47 and 76 % clonogenic cell killing respectively (Fig. 1), which suggests that these head and neck cell lines are more sensitive to AZT than MCF-7 or T47D cells, with FaDu being the most sensitive.

If mitochondrial dysfunction was responsible for the elevated production of $O_2^{\bullet-}$ and H_2O_2 seen in cancer cells, we predicted that inducing further mitochondrial dysfunction with AZT and CIS could kill cancer cells via metabolic oxidative stress. We did observe enhancement of cell killing in FaDu, Cal-27 and SQ20B head and neck cancer cells after concurrent treatment with 0.2 μ M CIS and 300 μ M AZT of both drugs for 24 h compared to either agent alone (Fig. 2A). We next analyzed changes in mitochondrial membrane potential which is a commonly used parameter to describe mitochondrial function. In healthy mitochondria, a proton gradient or potential is maintained across the mitochondrial membrane by electron transfer reactions. We observed that all three cell lines treated with AZT and CIS showed a significant loss in mitochondrial membrane potential (Fig. 2B), which suggests that disruption in mitochondrial membrane potential could contribute to the cytotoxicity of AZT and CIS shown in figure 2A.

When we analyzed the treated cells for glutathione content, we observed that oxidized glutathione (%GSSG) was significantly increased with AZT in combination with CIS (Fig. 2D). Glutathione is a major intracellular redox buffer such that the ratio of reduced glutathione to oxidized glutathione can be used as a reflection of intracellular redox status (29). The increase in % GSSG induced by AZT+CIS (Fig. 2D) suggests that oxidative stress is involved. These results support the speculation that AZT+CIS may have caused an increase in steady state levels of hydroperoxides and this increase exceeded the metabolic capabilities of the glutathione system to maintain glutathione in the reduced form. In fact, mitochondria are highly dependent on glutathione to detoxify ROS and prevent oxidative damage (31). To further support this idea, the thiol antioxidant NAC was able to inhibit the increase in %GSSG (Fig. 3D), reverse the loss of mitochondrial membrane potential (Fig. 3B) and partially inhibit the cytotoxicity induced by AZT+CIS (Fig. 3A). The AZT+CIS-induced disruption in mitochondrial membrane potential is indicative of mitochondrial effects that may be partially responsible for the increase we note in %GSSG by contributing to oxidative stress. The results with NAC show that mitochondrial potential and %GSSG are rescued when the cells are protected from toxicity (Fig. 3A,B). While this does not prove that the decrease in mitochondrial potential caused an increase in oxidative stress, it suggests that there may be a relationship between the two effects. Future studies will determine if this is indeed the case.

To further probe the role of glutathione in the effects of AZT+CIS, we used BSO, an inhibitor of glutamate cysteine ligase, which is believed to be the rate-limiting enzyme in the synthesis of GSH (37,38). Previous studies in our laboratory have shown that BSO significantly depleted glutathione pools in breast and head and neck cancer cells while sensitizing cancer cells to chemotherapy agents (39,40). BSO has also been used in clinical trials for cancer therapy to enhance the cytotoxicity of chemotherapeutic agents (41). In the present studies, BSO was

found to increase the cytotoxicity induced by AZT+CIS in our head and neck cancer cell lines (Fig. 4), which suggests that inhibition of glutathione synthesis sensitized these cells to the toxicity of AZT+CIS. Additionally, as proof of principle, NAC was shown to significantly inhibit the cytotoxicity induced by AZT+CIS+BSO in FaDu cells (Fig. 5). These results support the hypothesis that NAC is inhibiting the oxidative stress associated with AZT+CIS+BSO independent of GSH suggesting that NAC is acting to augment intracellular thiol antioxidants and inhibit thiol oxidation reactions that do not depend entirely on GSH synthesis.

Overall SQ20B cells were more resistant to AZT and/or CIS treatment than FaDu and Cal-27 cells (Fig. 1, 2). We also observed that BSO was not as effective in SQ20B cells as in FaDu and Cal-27 cells at sensitization to AZT+CIS (Fig. 4), which suggest that AZT+CIS-induced cytotoxicity in SQ20B cells is less dependent on glutathione than the other cell lines. We speculate that other antioxidant systems (i.e. thioredoxin) or other thiol oxidation reactions may be more important in SQ20B cells and warrant further investigation.

Cisplatin drug resistance in cancer cell lines has been shown to be reversed with AZT (42, 43). Postulated mechanisms of cisplatin drug resistance include decreased intracellular accumulation of cisplatin, increased intracellular levels of glutathione and increased DNA repair (44). Scanlon et al reported that AZT exposure to cisplatin resistant tumor cells restored cisplatin sensitivity by incorporation of AZT into DNA at sites where cisplatin-induced damage occurred (42). The AZT nucleotide subsequently results in chain termination of the DNA, failure of DNA repair, and resultant cell death (42). Because chemoresistance is a major obstacle in CIS therapy, the utility of AZT in combination with CIS has important clinical implications. Morgan et al 2003 showed in phase I trials that chemo-modulating doses of AZT were achievable over a 72 h period with minimal toxicity when administered in combination with CIS and demonstrated disease stabilization in patients previously non-responsive to CIS (45). Myelosuppression and neurotoxicity are known to be the dose limiting toxicities for AZT and CIS respectively. However, with the combination treatment, only myelosuppression was noted to be the dose limiting toxicity (45).

Our results shown in figure 4 suggest a potential rationale for the use of BSO in combination with AZT and CIS to treat cancer. BSO has been investigated in phase 1 clinical trials and showed minimal toxicity, with the predominant dose-limiting toxicity being nausea and occasional vomiting in patients that were previously exposed to platinum-based drugs, alkylating agents and taxanes (41,46). The use of BSO as an adjuvant and its normal tissue toxicity with the combination of AZT+CIS warrants further investigation.

The data provided here suggest that increased mitochondrial dysfunction and intracellular oxidative stress contributes to the toxicity of AZT+CIS, and manipulating intracellular thiol levels can affect cellular responses to AZT+CIS. Overall, studies with AZT and CIS suggest that these compounds are capable of disrupting mitochondrial function, increasing oxidative stress as well as causing significant cytotoxicity in head and neck cancer cells that appears to be partially mediated by disruptions in thiol metabolism. These data provide a potential rationale for the use of mitochondrial directed agents in combination with conventional chemotherapeutic drugs and inhibitors of glutathione metabolism in the clinical setting of cancer therapy.

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List of Abbreviations

CIS	cisplatin
AZT	zidovudine
BSO	L-buthionine-[S,R]-sulfoximine
NAC	N-acetylcysteine
GSH	glutathione
GSSG	glutathione disulfide

Figure 1.

Effect of zidovudine (AZT) on survival of FaDu (black bars), Cal-27 (hatched bars) and SQ20B (white bars) cells. The cells were treated with 10 – 1000 $\mu\text{mol/L}$ AZT for 24 h then plated for clonogenic survival. Clonogenic cell survival data were normalized to control (CON). Error bars represent $\pm$ standard error of the mean of N=3 experiments performed on different days with at least 4 cloning dishes taken from 1 treatment dish. *, $p < 0.05$ versus respective control.

Figure 2.

Effect of zidovudine (AZT) and cisplatin (CIS) on cytotoxicity (A), mitochondrial membrane potential (B), total glutathione (GSH) levels (C) and percentage oxidized glutathione (% GSSG) levels (D) in FaDu (black bars), Cal-27 (hatched bars) and SQ20B (white bars) cells. A: Cells were treated with 300 μ M AZT and/or 0.2 μ M CIS for 24 h. Clonogenic cell survival data were normalized to control (CON). B: Cells were treated as stated above, labeled with JC-1 dye for 15 min and analyzed by flow cytometry. C,D: Cells were treated as stated above and harvested for glutathione analysis using the spectrophotometric recycling assay. Error bars represent $\pm$ standard error of the mean of 2-3 experiments. *, $p < 0.001$ versus control; ¥, $p < 0.001$ versus AZT or CIS alone.

Figure 3.

Effect of zidovudine (AZT) and cisplatin (CIS) and N-acetyl-cysteine (NAC) on cytotoxicity (A), mitochondrial membrane potential (B), total glutathione (GSH) levels (C) and percentage oxidized glutathione (% GSSG) levels (D) in FaDu (black bars) and Cal-27 (hatched bars) cells. A: Cells were treated with 300 μ M AZT and 0.2 μ M CIS for 24 h with or without treatment with 20 mM NAC for 1 h before and during AZT and CIS exposure. Clonogenic cell survival data were normalized to control (CON). B: Cells were treated as stated above, labeled with JC-1 dye for 15 min and analyzed by flow cytometry. C,D: Cells were treated as stated above and harvested for glutathione analysis using the spectrophotometric recycling assay. Error bars represent $\pm$ standard error of the mean of N=3-7 experiments. *, $p < 0.001$ versus control; ¥, $p < 0.001$ versus respective treatment without NAC.

Total GSH (nmoles)	14.0±2.4	1.1±0.2*	13.9±3.2	2.0±0.9*	<i>FaDu</i>
	21.5±1.3	0.1±0.2*	22.0±5.5	0.1±0.1*	<i>Cal-27</i>
	20.0±3.5	2.6±0.1*	12.1±2.0	2.7±0.2*	<i>SQ20B</i>

Figure 4.

Effect of zidovudine (AZT) and cisplatin (CIS) and L-buthionine-[S,R]-sulfoximine (BSO) on cytotoxicity in FaDu (black bars), Cal-27 (hatched bars) and SQ20B (white bars) cells. Cells were treated with 300 μ M AZT and 0.2 μ M CIS for 24 h with or without treatment with 1 mM BSO for 1 h before and during AZT and CIS exposure. Clonogenic cell survival data were normalized to control (CON). Cells were harvested for glutathione analysis using the spectrophotometric recycling assay. Error bars represent $\pm$ standard error of the mean of N=3 experiments performed on different days with at least 4 cloning dishes taken from 1 treatment dish. *, $p < 0.001$ versus control; ¥, $p < 0.05$ versus respective treatment without BSO.

Figure 5.

Effect of N-acetyl-cysteine (NAC) on zidovudine (AZT), cisplatin (CIS) and L-buthionine-[S,R]-sulfoximine (BSO)-induced cytotoxicity in FaDu cells. Cells were treated with 1 μ M BSO, 300 μ M AZT and 0.2 μ M CIS for 24 h with or without treatment with 20 mM NAC for 1 h before and during AZT, CIS and BSO exposure. Clonogenic cell survival data were normalized to control (CON). Error bars represent $\pm$ standard error of the mean of N=3 experiments performed on different days with at least 4 cloning dishes taken from 1 treatment dish. *, $p < 0.001$ versus control; ¥, $p < 0.05$ versus respective treatment without NAC.