Acetaminophen and DMSO modulate growth and gemcitabine cytotoxicity in FM3A breast cancer cells *in vitro*

A. BILIR[1], A.D. GUNERI[1], M.A. ALTINOZ[2*]

[1]Department of Histology and Embryology and [2]Department of Neuroimmunology, e-mail: maltinoz@hotmail.com, Montreal Neurological Institute and Hospital, Montreal, H3A 2B4, Quebec, Canada

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Addition of antioxidants to chemotherapy is an unresolved problem in oncology. It is still an issue of debate, whether antioxidants may reduce rough cellular toxicity and thereby the systemic side effects of the chemotherapy, without sacrificing the anti-tumor efficacy.

Gemcitabine is a rather new anti-cancer agent, which is quite potent against a range of drug resistant tumors, particularly breast cancer. Tumor-sensitivity towards gemcitabine can be increased with COX inhibitory anti-inflammatory agents and ribonucleotide reductase (RR) inhibitor flavopiridol. Acetaminophen and DMSO are two unique anti-inflammatory and anti-oxidant agents with unrelated structures, yet both capable to block RR and COX, simultaneously. Using plating efficacy and ^{3}H-thymidine labeling, we monitored efficacy of acetaminophen and DMSO to modulate growth and gemcitabine sensitivity in FM3A breast tumor cells, which is highly used to study thymineless death induced by nucleotide-metabolism hemming drugs. Peculiarly, acetaminophen alone stimulated S-phase, which was not accompanied with enhanced plating, rather resulting in 40.3% growth inhibition at the 96 hour. DMSO alone significantly diminished both the plating and S-phase, which resulted in 71.7% growth inhibition at the 96 hour. Gemcitabine drastically reduced S-phase and plating until 72 hours, yet at 96 hours it lost its efficacy to suppress the S-phase with concomitant 2-fold rise in cell numbers in comparison to 72 hour time point. Both DMSO and acetaminophen brought S-phase to around zero percent in combination with gemcitabine until 48 hours, yet they both reduced early cytotoxicity of gemcitabine at the same time interval. However, at the 96 hour, they both strongly augmented gemcitabine efficacy to block S-phase and prevented the rise in plating. Acetaminophen and DMSO should be tested in animal models, whether they could augment efficacy and reduce the toxicity of gemcitabine.

Key words: acetaminophen, DMSO, gemcitabine, breast cancer, FM3A

Gemcitabine (2',2'-difluorodeoxycytidine) is a nucleoside analogue with promising activity on many solid tumors with particular importance in breast cancer [23]. It blocks tumor growth via irreversible inhibition of DNA polymerase epsilon [15], and suppression of ribonucleotide reductase (RR) [23]. Since there is a correlation between expression of cyclooxygenase (COX) and P-glycoprotein in breast cancers [22], it has been hypothesized that COX inhibitory drugs could overcome drug resistance before the development of resistant tumor clones [22]. Thus, we aimed to study modulation of long term cytotoxic (plating) and cytostatic (S-phase) effects of gemcitabine with antiinflammatory drugs.

Acetaminophen reduces the active oxidized form of COX to the resting form, and thereby inhibits its activity [20], moreover it reduces DNA synthesis by a specific inhibition of RR [14]. DMSO also inhibits prostaglandin biosynthesis from eicosa-8, 11, 14-trienoic acid in bovine seminal vesicles [21]. It is also widely recognized that DMSO shows potent anti-tumor activity via induction of differentiation, and during induction of granulocytic differentiation in HL-60 promyelocytic leukemia cells, it completely inhibits the expression of the M2 subunit of RR [4].

*Author to whom correspondence should be sent.

Therefore, DMSO and acetaminophen were selected as candidate drugs for gemcitabine sensitization, since they may block both RR and the proclaimed co-mediator of drug resistance, COX. This study is also an extension to our previous research focus, in which we showed that acetaminophen could be used for carboplatin sensitization in human ovarian cancer cells [1].

Material and methods

Cell culture and drugs. The FM3A cell line established from the American Type Culture Collection was maintained in RPMI-1640 medium (Biological Industries Haemek, Israel) with 10% heat-inactivated fetal calf serum, 100 μg/ml penicillin and 100 μg/ml streptomycin. Flasks were kept in an incubator with a humidified atmosphere of 5% CO_2 at 37 ˚C. Cells were transferred using Ca- and Mg-free Hanks' basic salt solution and 0.25% trypsin-EDTA (Biological Industries). Acetaminophen (A3035, Sigma), DMSO (D2650, Sigma) and Gemcitabine (Gemzar^R, Lilly) were dissolved in bidistilled water and added into cell culture in equal volumes.

Plating efficacy and cytotoxicity. Exponentially growing cells prepared in 5 ml of RPMI-1640 were plated to each well of a6-well plate at a concentration of $1x10^6$/ml with 100% viability. Drugs were added in equal volumes of 100 μl to the plates, after cell harvesting. Cell viability was estimated by counting the number of cells, which excluded a trypan-blue solution (in 0.4% normal saline; Gibco) in a hemocytometer after each 24-hour period of plating until the 96th hour. The viability of the controls was always more than 90%.

^{3}H-Thymidine labeling assay. Half an hour before the end of each 24-hour period of monitoring of the plating, cells were incubated with $37x10^3$ Bq/ml H-thymidine (specific activity 185 GBq/mmol, Amersham, England). Following 30 minutes of incubation (pulse labeling), cells were trypsinized and smear slides were prepared. Then the slides were fixed with Carnoy's fixative (ethanol-glacial acetic acid at a ratio of 3:1). Inbound radioactive materials were washed twice with 2% perchloric acid at 4 ˚C for 30'. To determine the labeling index, cover slips were coated with gel emulsion film (Ilford K2, England).

After 3 days of exposure at 4 ˚C, autoradiograms were washed with D-19 developer and all the slides were evaluated by the same person after Giemsa staining. On each slide, at least $3x10^3$ cells were evaluated in 100 different areas with 100x12.5 magnification; cells with at least 5 grains in their nuclei were considered to be labeled, and background grain was defined as less than 1 per area. Thymidine labeling indices (TLIs) were determined by division of the thymidine labeling value of each treatment group by the value of the thymidine labeling of the controls.

Results

Plating efficacy (and percentage of trypan blue positive cells). As shown in Figure 1a, cells in the control group exerted a healthy growth beginning with approximately 0.9 million cells at 24 hours, rising to 9.1 million cells at the 96 hour. Acetaminophen at all times reduced the cell numbers, which occurred with 40.3% inhibition at the 96 hour, and these reductions were statistically significant for the 48, 72 and 96 hour time points with $p<0.05$, $p<0.01$ and $p<0.05$, respectively. DMSO was at all times more potent than acetaminophen to inhibit plating, which reached to 71.7% growth inhibition at the 96 hour, and which were always significant with $p<0.05$, $p<0.00001$, $p<0.005$ and $p<0.005$ for 24, 48, 72 and 96 hour time points, respectively. Gemcitabine drastically reduced cell numbers very significantly at all intervals, which occurred at 98.4% inhibition at the 96 hour, and with $p<0.01$, $p<0.005$, $p<0.005$, $p<0.005$ for 24, 48, 72 and 96 hours, respectively. Acetaminophen at the 24 and 48 hours slightly reduced gemcitabine cytotoxicity, which occurred with significance at the 48 hour ($p<0.05$). However, on the 72 hour and 96hour time points, it further reduced cell numbers by 41.0% and 46.0% in comparison to gemcitabine alone, which was significant for the latter ($p<0.05$). DMSO acted very similar like acetaminophen, and except the 48 hour time point, it always further reduced cell numbers in comparison to gemcitabine alone, which reached to 64% further reduction of the cell number at the 96 hour and which occurred with $p<0.05$ and $p<0.005$ for 24 and 96 hour time points, respectively. Since the cell numbers with gemcitabine are quite low in comparison to control, the modifications on gemcitabine efficacy by DMSO and acetaminophen are depicted in a different graph in Figure 1b, where the cytotoxicity of gemcitabine is diminished by both DMSO and acetaminophen until the first 48 hours and augmented thereafter. When we separately graphed only the trypan-blue positive necrotic cells, it became obvious that the efficacy of DMSO and acetaminophen to reduce early cytotoxicity of gemcitabine is mainly via prevention of cellular necrosis. Moreover, a reduced dose of acetaminophen seemed to be less effective in reducing cellular necrosis at the 48 hour time point.

^{3}H-Thymidine labeling index (TLI) / S-phase fraction. Control group cells had a healthy pattern of S-phase fraction, which reduces in time due to enhanced population of cells consuming from the same nutritional pool, with TLI values of 41.7%, 48.9%, 23.9%, 12.9% at 24, 48, 72 and 96 hours, respectively. Acetaminophen at al times increased the TLI values to 52.1%, 54.3%, 44.2% and 37.5% at 24, 48, 72 and 96 hours, respectively. All these changes were highly significant with $p<0.000001$, $p<0.001$, $p<0.000001$ and $p<0.000001$ for 24, 48, 72 and 96 hours, respectively. DMSO

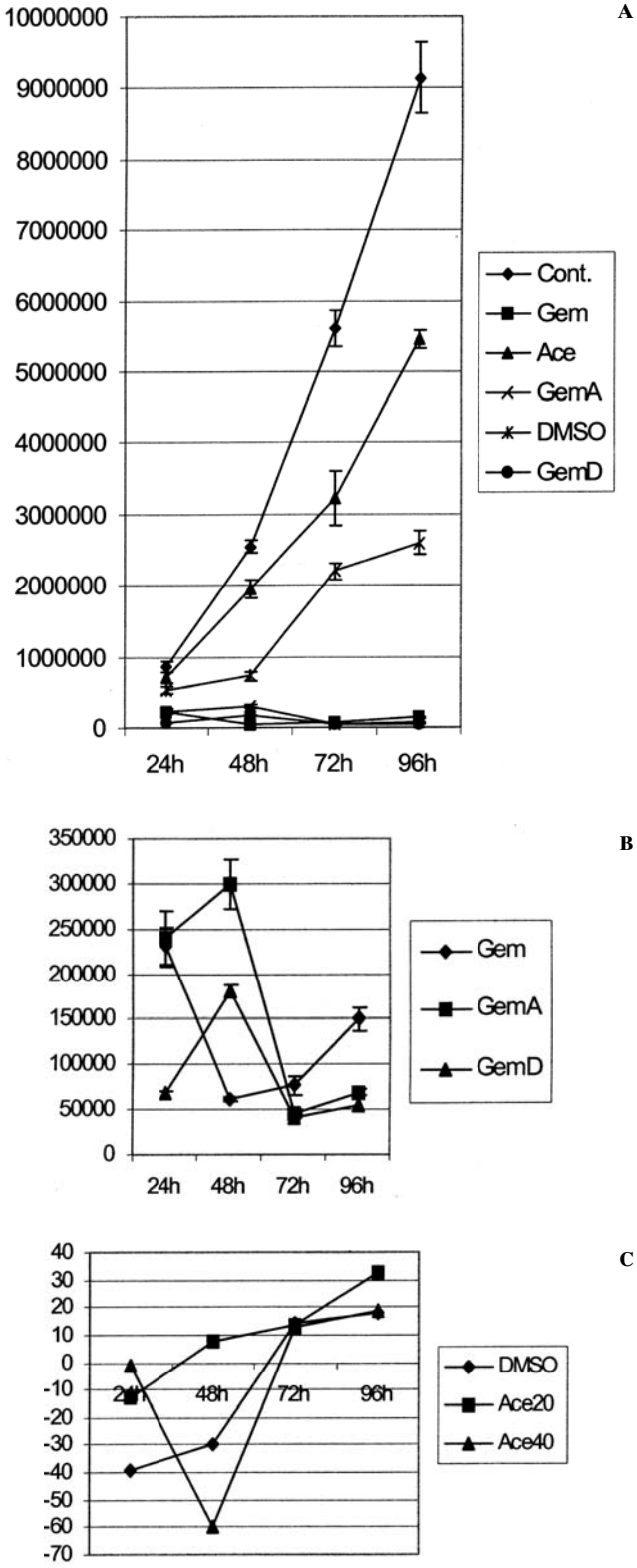

Figure 1. a) Modulation of FM3A cell plating with gemcitabine and/or DMSO or acetaminophen; b) Plating efficacy of FM3A cells [gemcitabine treated groups only]; c) Modulation of gemcitabine induced trypan blue positive cells with DMSO and acetaminophen.

reduced the TLI values very significantly until 72 hours, yet it lost its efficacy to suppress the TLI at 96 hour, more so because the control group TLI declined sharply after 48 hours. It brought TLI values to 11.1%, 15.7%, 15.5% and 15.5% at 24, 48, 72 and 96 hours, respectively. These changes occurred always with high significance value with $p<0.000001$ until 72 hours, and the slight higher TLI at 96 hour in comparison to control was statistically insignificant ($p>0.05$). Gemcitabine drastically reduced TLI values to 3.9%, 1.9% and 0.0% at 24, 48 and 72 hours, respectively, which always occurred with very high significance ($p<0.000001$). However, remembering the completely blocked S-phase at 72 hours, a dramatic change occurred at the 96 hour time point, when the remaining vital cells again underwent into an active cell cycling with a TLI value of 10.6%, which was not statistically different from control ($p>0.05$), indicating complete loss of gemcitabine efficacy at this time point. Parallel to this data trypan blue excluding vital cell number was double at 96 hour in comparison to 72 hour cell numbers in gemcitabine-treated group, as given above. When acetaminophen was combined with gemcitabine, except the 72 hour time point, it always augmented gemcitabine efficacy to suppress the S-phase with $p<0.00001$ and brought TLI values to 0.0%, 0.0%, 2.02%, and 1.3%, for 24, 48, 72 and 96 hours, respectively. DMSO acted very similar like acetaminophen, and except the 72 hour time point it also very strongly ($p<0.000001$) augmented TLI suppressions to 0.3%, 0.0%, 0.1% and 0.3% at 24, 48, 72 and 96 hours, respectively.

Discussion

Gemcitabine was very active in reducing the S-phase and plating of FM3A cells until the 72 hour in our study. But thereafter, inhibition of S-phase has ceased, and plated cell number has doubled in comparison to cell numbers at 48 and 72 hours, which were at steady-state. We postulate that a small cell colony from the whole cell population have lost their sensitivity towards gemcitabine during continuous exposure. Degradation of gemcitabine in culture media is less likely, since co-presence of either DMSO or acetaminophen prevented the rise in cell number and obtained very large decreases in S-phase at the 96 hour, such as seen with single gemcitabine at earlier intervals.

Since neither DMSO and nor acetaminophen had such a potential in their single action, it is logical to assume that they have prevented the emergence of an insensitive cell population.

Acetaminophen role as COX blocking agent is well defined [20], and DMSO could also suppress prostaglandin synthesis [21]. It should be elucidated, whether these drugs may have prevented formation of insensitive clones because of suppressing the COX-product dependent cells, which

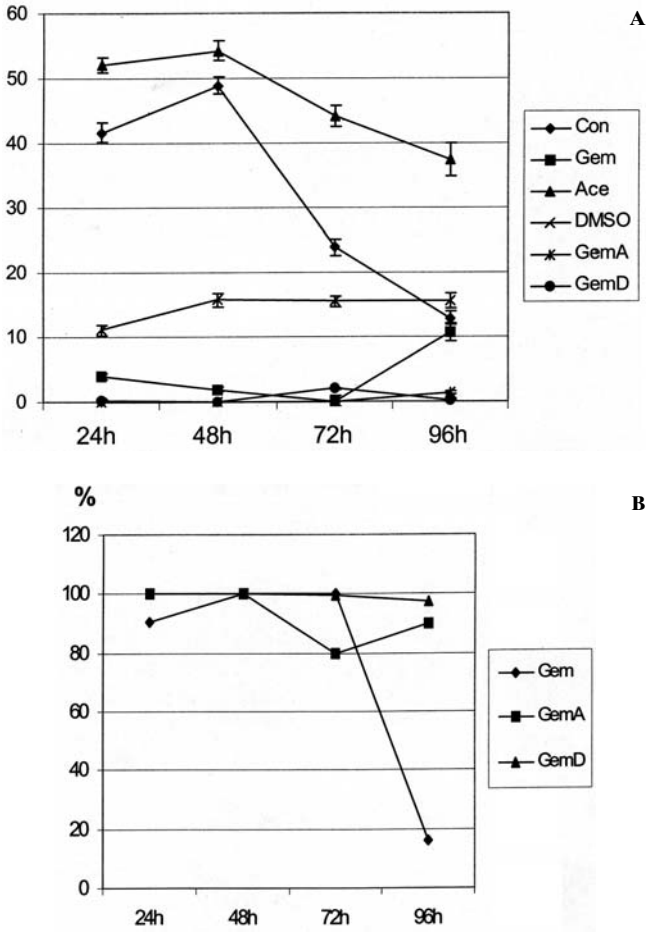

Figure 2. a) Thymidine labeling indices of FM3A cells treated with gemcitabine and/or DMSO or acetaminophen; b) Modulation of gemcitabine depletion of thymidine labeling with acetaminophen and DMSO.

simultaneously may have expressed drug excluding and anti-apoptotic P-glycoprotein [22].

Instead of waiting the gain of resistance by cancer cells via repeated drug applications, the drugs were applied simultaneously in our study. As indicated above, this condition was our main drive to think that the late interval drug sensitization by DMSO and acetaminophen were prevention rather than reversal of drug resistance. Drug resistance is a major problem of cancer chemotherapy and occurs via multiple mechanisms including enhanced efflux of chemotherapeutical drugs by P-glycoprotein dependent and non-dependent mechanisms, transcriptional activation of certain genes, or enhanced DNA repair involving DNA topoisomerases [19]. Among these, some transcription-activated proteins force multiple pathways towards enhanced levels of intrinsic drug resistance and tumor aggressiveness.

Ribonucleotide reductase (RR) enzyme is one of these, and its overexpression is a major mechanism of gemcitabine resistance [16]. It first gained attention as chemotherapeutical target, since it catalyzes synthesis of dNTP' s as substrates for DNA replication [6]. Then it was found that the enhanced RR activity in rat myoblast cell lines was correlated with reduced cell differentiation [7]), and the reduced RR activity with cell senescence [8]. Currently it is recognized that elevation of the RR activity, and particularly its small M2 subunit is correlated with enhanced de-differentiation and even with metastatic phenotype of cancer cells [5].

Differentiation agents such as DMSO [4] and Vitamin D3 [3] could induce dramatic and long-lasting reductions of M2 subunit expression during induction of HL-60 leukemia cell differentiation. We think that M2 subunit of RR is also a likely target by DMSO and acetaminophen to prevent gemcitabine insensitivity of FM3A cells at 96 hour interval in our study, since both inhibit RR, as mentioned [4, 14]. Moreover, a recent study has showed that flavopiridol, a cyclin dependent kinase inhibitor, could very potently enhance gemcitabine induced clonogenic and apoptotic cell death in gastrointestinal cancer cells, with transcriptional down-regulations in M2 subunit of RR [16]. Other targets, such as inhibition of NF-KB may also underlie gemcitabine sensitizing effects of anti-inflammatory drugs, since NSAID' s have found to sensitize pancreas cancer cells towards gemcitabine independently of COX expression status [28]; and both acetaminophen [2] and DMSO [17] could inhibit NF-KB, a transcription factor, which is selectively involved in breast cancer drug resistance [27]. Inhibition of NF-KB would also explain some of the findings of our study, which seem paradoxical at the first glance. Acetaminophen alone has increased S-phase of FM3A cells, but this stimulation was not reflected by the plated cell numbers. We have made a similar observation of S-phase elevation in MDAH 2774 human ovarian cancer cells with acetaminophen, despite inducing carboplatin sensitization [1].

These investigations are parallel to a series of studies by HARNAGEA-THEOPHILUS et al, which showed that acetaminophen induces breast cancer cell proliferation through interaction with estrogen receptors but without binding to them [11, 12, 13].

The uncoupling of S-phase elevation from enhancement of cellular plating may relate with NF-KB inhibitory effect of acetaminophen [2], since NF-KB plays a significant role in enhanced cellular adhesion [25]; and loss of NF-KB activity reduces CD11b integrin [25], which is involved in plastic surface adherence [9]. Lastly, we will discuss modulation of rough toxicity of gemcitabine by acetaminophen and DMSO. At earlier intervals until 48 hour, both DMSO and acetaminophen prevented cellular necrosis induced by gemcitabine, but thereafter both drugs enhanced it. Despite that we did not measure apoptosis, we presume that enhanced necrosis seen with these drugs at later periods may be result of secondary necrosis of apoptotic bodies.

Both acetaminophen [18] and DMSO [24] possess significant antioxidant properties, these may have helped to reduce rough gemcitabine toxicity at earlier intervals; and

may also help to reduce chemotherapeutic toxicity at a clinical setting. Though higher doses of acetaminophen may act pro-oxidant through its metabolite N-acetylbenzoquinonimine [10], it is less likely to be causal factor for enhanced late interval necrosis with acetaminophen-gemcitabine combination here, since higher dose (40 μg/ml) of acetaminophen induced less rough toxicity then the lower (20 μg/ml) dose, probably due to a higher antioxidant effect. Moreover, at the clinical stage, directing pro-oxidant metabolism of acetaminophen in tumor cells rather than in hepatocytes is now possible via selective activation of the cytochrome CYP2D6 in the tumor mass by gene therapy [26].

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