Modulation of Gemcitabine Accumulation by DNA-Damaging Agents: Mechanisms and Specificity in an In Vitro Model

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Abstract. Self-potentiation of the intracellular accumulation of gemcitabine accumulation occurs with repeated administration. Understanding the mechanism of this phenomena and its occurrence with other drugs is important for rational dosing of gemcitabine and design of gemcitabine combinations. The HCT116 cell line was used as a model of the in vivo findings to examine the effect of repeated gemcitabine exposure. HPLC analysis revealed a 10-fold increase in gemcitabine-triphosphate accumulation upon repeated gemcitabine exposure. The induction of accumulation was not associated with any changes in the dCK mRNA level. Comparable increases in gemcitabine-triphosphate were seen when the cells were pre-incubated with cytarabine and cisplatin. A lesser increase and no increase in GEM-TP were seen with oxaliplatin and 5’-azacytidine, respectively. In this model, induction of gemcitabine accumulation is likely to be mediated by post translational modification of dCK. The reduced effect of oxaliplatin compared to cisplatin is worthy of further study.

Self-potentiation of gemcitabine (GEM) is central to its pharmacological advantage and is well recognized as an in vitro observation. It has been previously demonstrated that gemcitabine induces its own accumulation in clinical use, resulting in an increasing ability to accumulate in white blood cells with repeated dosing (1). It is important to understand the mechanism of this increase because it potentially impacts on the combination of gemcitabine with other drugs. There are multiple potential mechanisms of this auto-induction, including alterations in transport, nucleotide depletion caused by inhibition of RRM1, and alteration in dCK activity. There is evidence that other DNA-damaging agents are able to cause an increase in dCK mRNA expression or/and an increase in dCK activity (2-6). The induction is likely to be associated with an increase in dCK activity, because dCK is the rate-limiting enzyme for GEM-TP accumulation (7). A possible cause of this induction is an increase in dCK mRNA expression which in turn could increase the dCK activity initiated by the effect of GEM exposure.

The aims of this work were (i) to establish a cell line model that can be used to demonstrate an induction of GEM-TP accumulation, (ii) to compare the effect of GEM with other DNA-damaging agents in inducing GEM accumulation, and (iii) to explore potential mechanisms by evaluating changes in dCK mRNA expression.

Materials and Methods

Cell culture. The HCT116 cell line was maintained in a culturing flask at 37°C in an atmosphere of 95% air, 5% CO2, and 99% relative humidity in MEM (Sigma, Australia) supplemented with 10% (v/v) FCS (JRH Biosciences, Kansas, USA) and penicillin/streptomycin with 200 mM L-glutamine (Sigma, Australia) to a final concentration of 50 μg/ml. Only exponentially growing cells and mycoplasma-free cells were used to set up experiments.

Preparation of drug solution. Cytotoxic drugs used in this study were prepared as stock solutions. The stock solution of GEM (Eli Lilly, Indianapolis, USA) was 2 mM (0.6 mg/ml), 1 mM for 5’-azacytidine (5’-azaC) (0.24 mg/ml) (Sigma, Australia) and 1 mM for cytarabine (Ara-C) (Sigma, Australia) in FCS-free MEM medium, 0.5 mM cisplatin (0.15 mg/ml) (Sigma, Australia) and 1 mM of oxaliplatin (0.4 mg/ml) (Sigma, Australia) was prepared in sterile PBS. Stock drug solutions were divided into aliquots and stored at ~20°C.

Determination of GEM-TP accumulation. In order to determine the ideal concentration of GEM and drug incubation time for the study of auto-induction of GEM metabolism, it was necessary to determine the ability of HCT116 cell to accumulate GEM-TP. The cells were incubated with 50 μM GEM, a concentration of GEM similar to the maximum plasma GEM concentration (plasma Cmax) demonstrated in patients in a previous study (1), for up to 6 h. The
GEM-TP was extracted from the cells at intervals of 30 min, 1, 2, 3, 4, 5 and 6 h and was quantified by ion-exchange HPLC. Three independent experiments were performed. 

**Extraction of GEM-TP for HPLC.** Approximately 5x10⁶ cells were harvested and resuspended in 5 ml ice-cold PBS in a 10 ml centrifuge tube. The cell suspension was centrifuged at 250 g for 10 min at 4°C. The supernatant was removed and the cell pellet was resuspended in 510 μl PBS. Cell suspension was mixed with 7% ice-cold perchloric acid (Sigma, Australia) in a ratio of 1:1 (v/v). The mixture was neutralized to a target pH of 7.0 with 2 M NaOH. The mixture was centrifuged at 16000xg for 10 min at 4°C. The supernatant containing the GEM-TP was transferred to a new microfuge tube and stored at −20°C until ready to be analysed by HPLC. The number of viable cell was counted by trypan blue exclusion assay. 

**HPLC conditions for GEM-TP quantification.** Solutions of GEM-TP and NTPs at a concentration of 0.5, 1, 2, 5, and 10 μM were used to prepare the HPLC calibration curve for GEM-TP measurement. Mobile phases were used in this gradient HPLC. The mobile phase ‘A’ was NH₄H₂PO₄ (Sigma, Australia) at 0.005 M and pH 2.8. Mobile phase ‘B’ was NH₄H₂PO₄ at 0.75 M and pH 2.8. Water and mobile phases were passed through a 0.45 μm cellulose nitrate membrane filter (Whatman, Kent, UK) before used. The gradient was run over 40 min with 10 min equilibration time to give a total run time of 50 min per sample on a Shimadzu HPLC system (Kyoto, Japan) comprising of a gradient pump (model LC-10Ai), an automated injector (model SIL-10A), a degasser unit (model DGU-14A), a UV detector (model SPD-10A VP), and a system controller (model SCL-10A). The gradient was 0% mobile phase B to 100% mobile phase B over 40 min. A total sample volume of 200 μl was injected into a 2 ml sampling loop. The UV wavelength used for detection was set to 272 nm. An ion-exchange PARTISIL 10 SAX (4.6x250 mm, 10 μm) column (Whatman, Kent, UK) was used.

**Repeated exposure of the cell line to GEM.** To evaluate the effect of repetitive exposure to GEM on GEM-TP accumulation, the cells were incubated with 50 μM GEM for 30 min twice with a 24 h drug-free incubation in cell culturing medium in between both exposures. The GEM-TP and dCK mRNA transcript were measured at 5 different time-points (T0-T4) in the experiment: T0, no drug control (prior to first incubation); T1, after the first GEM incubation; T2, 24 h post T1 (prior to second incubation): T3, after second GEM incubation; and T4, 24 h after second GEM incubation. GEM-TP extraction and quantification methods were as described above. Three independent experiments were performed. The GEM-TP accumulation is presented as mean±SD of three independent experiments.

**mRNA extraction and cDNA synthesis.** Approximately 5 - 10x10⁶ cells were harvested from the cell-culture, flowered and resuspended in 5 ml of ice-cold PBS in a centrifuge tube. The cell suspension was centrifuged at 250xg for 5 min. The mRNA extraction was carried out using Trizol reagent (Invitrogen, Australia) according to the manufacturer’s protocol. Varied amounts of mRNA template were used for cDNA synthesis. The amounts of mRNA reverse transcribed for the set up of β-actin standard curve were 1 μg, 0.1 μg, 10 ng, 1 ng , and 0.1 ng. For the dCK standard curve, the amounts of mRNA reverse transcribed were 1 μg, 0.5 μg, 0.25 μg, 0.125 μg, and 62.5 ng. The cDNA used in the quantitative real-time PCR (qRT-PCR) was synthetised from 0.5 μg mRNA extracted from the cells at each specific time-points (T0-T4). The 20 μl reverse transcription reaction consisted of 2 μl of mRNA, 1 μl of Oligo(dT)12-18 (Invitrogen), 1 μl of 2 mM dNTP mix (Fermentas Life Science, Ontario, Canada), 4 μl of 5 x first strand buffer, 2 μl of 0.1 M DTT, 1 μl of RNaseOUT ribonuclease inhibitor (40 U/μl) (Invitrogen, Australia), 8 μl of filtered sterile MilliQ water, and 1 μl of Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (200 U/μl) (Invitrogen, Australia). The reaction condition was carried out according to M-MLV RT manufacturer’s protocol.

**Quantitative real-time PCR.** cDNA synthesized from the earlier step was used to set up the qRT-PCR. The method of qRT-PCR used was as previously described by Pfaffl (8). The quantitative method did not require the internal standard curve in every qRT-PCR run. Three independent standard curves were established for each gene. The mean reaction efficiency was calculated from three standard curves. The cycling conditions were adjusted for the amplification of β-actin and dCK so that the reaction efficiency was closest to 1 (100% amplification) and with the standard error of three independent runs of less than 0.01. The PCR efficiency was used to calculate relative mRNA expression at different time-points from the equation described by Pfaffl (8).

**Different cycling temperature profiles were required for β-actin and dCK qRT-PCR.** The cycling condition for β-actin qRT-PCR consisted of 2 min hold at 50°C and 90°C followed by 40 cycles of 95°C for 5 s, 57°C for 10 s, and 72°C for 10 s, and followed by melt analysis with the temperature ramp from 50-95°C, raising by 1°C for each step. The cycling condition for dCK qRT-PCR was similar to the β-actin but the 40 cycles consisted of 95°C for 5 , 58°C for 10 s, and 72°C for 10 s. The fluorescent detection of the light cycler (Corbett Life Science, Sydney, Australia) was set at 510 nm. The 20 μl qRT-PCR reaction consisted of 2 μl of cDNA (0.25 μg/ml), 10 μl SYBR Green qPCR supermix (Invitrogen, Australia), 1 μl 20x bovine serum albumin (BSA) (Invitrogen, Australia), 1 μl of 10 μM forward primer (β-actin forward 5’-AGAGCTACGAGCTGC CTCGAC-3’); or dCK forward 5’-AGACGCTGTGCGCTGA CAG-3’), 1 μl of 10 μM reverse primer (β-actin reverse 5’-GATT GGAAAGTGCTTCCTGA-3’; or dCK reverse 5’-GAGCTTGGCA TTCAGAGAGG-3’) (synthesized by Genosys, Sigma, Australia), and 5 μl filtered sterile MilliQ water.

**Effect on the GEM-TP accumulation in cells pre-incubation with other anticancer drugs.** The cells were pre-incubated with other anticancer drugs prior to GEM incubation. The concentration of cytotoxic drugs used in this experiment resembled the plasma Cmax of drugs reported in patients from clinical studies (9-12). Two nucleoside analogues and two platinum drugs used in this study: Ara-C, 5’-azaC, cisplatin, and oxaliplatin, respectively. To study the effect of other anticancer drugs on GEM-TP accumulation, cells were exposed to either 20 μM Ara-C, 3 μM 5’-azaC, 30 μM cisplatin, or 4 μM oxaliplatin for 100 min. The cells were washed twice with PBS and replenished with fresh MEM medium and were cultured for another 24 h followed by incubation with 50 μM GEM for 3 h. The intracellular GEM-TP accumulation was measured by HPLC at the end of GEM incubation. The amount of intracellular GEM-TP accumulation was compared with the level of GEM-TP accumulation of the control (CTRL=3 h 50 μM GEM without pre-incubation to other anticancer drug). The level of GEM-TP
accumulation was also compared with repeated GEM incubation where the cells was pre-incubated with 50 μM GEM for 30 min prior to 50 μM GEM incubation for 3 h. The intracellular GEM-TP level was presented as mean±SD of three independent experiments.

Statistics. All data are presented as mean±standard deviation. The statistical significant of data was determined by paired Student’s t-test test of equal variance using Graphad Prism version 4 (Graphad Software, CA, USA) on a desktop computer. P-values of less than or equal to 0.05 were considered statistically significant.

Results

Establishing a model of GEM auto-induction. GEM-TP accumulation was linear up to 6 h from 108±28 pmol/10^6 cells (30 mins) to 976±56 pmol/10^6 cells (6 h) after a continuous incubation to GEM (data not shown).

The levels of intracellular GEM-TP accumulation in HCT116 cells after repeated exposure are illustrated in Figure 1. GEM-TP accumulation after the first round of GEM exposure (T1) was 58±87 pmol/10^6 cells. At 24 h after first GEM incubation (T2), the GEM-TP decreased to a level that could not be detected by HPLC. After the second drug exposure (T3), there was more than a 10-fold increase in GEM-TP accumulation (p=0.02), with a GEM-TP level of 757±120 pmol/10^6 cells. At 24 h post second incubation to GEM, the GEM-TP was detectable, the intracellular GEM-TP retention was higher than that at 24 h post first GEM incubation, with a GEM-TP level of 113±72 pmol/10^6 cells.

Auto-induction is not due to changes in dCK mRNA. Figure 2 illustrates the relative dCK mRNA expression at different time-points in the experiment. The assays were carried out in triplicate. There was no significant change in dCK mRNA expression after the first exposure to GEM (T1), prior to second exposure to GEM (T2) or in the 24 h after the second drug exposure (T3-T4).

Effect of pre-incubation of cells to other DNA damaging agents on GEM-TP accumulation. Nucleoside analogues: 5’-azaC and Ara-C. Figure 3 illustrates the level of intracellular GEM-TP accumulation in HCT116 cell line. There was a 1.4-fold increase in GEM-TP accumulation in the cells pre-incubated with 5’-azaC (p=0.17) and a 7-fold increase in GEM-TP accumulation in cells pre-incubated with Ara-C compared to the control (p=0.03), with a mean±SD GEM-TP of 818±116, 4175±1200 and 597±70 pmol/10^6 cells, respectively. The level of GEM-TP accumulation in the cells that were pre-incubated with Ara-C was similar to that in the cells that were pre-incubated with GEM (p=0.05). The GEM-TP accumulation in cells that were pre-incubated with 5’-azaC was 5-fold less than in the cells that were pre-incubated with GEM (p=0.04).

Platinum drugs: cisplatin and oxaliplatin. The ability of HCT116 cells to accumulate GEM-TP after pre-incubation with platinum drugs is illustrated in Figure 4. The cells pre-incubated with cisplatin accumulated 5-fold more GEM-TP than the control (p=0.002), with a GEM-TP level of 2895±240 pmol/10^6 cells and 597±70 pmol/10^6 cells, respectively. The level of GEM-TP accumulation in cells pre-incubated with oxaliplatin was similar to the control (p=0.1) with a GEM-TP level of 658±120 pmol/10^6 cells and 597±70 pmol/10^6 cells, respectively. The cells that were pre-incubated with cisplatin accumulated 2.6-fold more GEM-TP than the cells that were pre-incubated with oxaliplatin (p=0.01). The level of GEM-TP accumulation in the cells that were pre-incubated with cisplatin was similar that in the
cells that were pre-incubated with GEM ($p>0.05$). The GEM-TP accumulation in cells that were pre-incubated with oxaliplatin was 4-fold less than the cells that were pre-incubated with GEM ($p=0.04$).

**Discussion**

The previously published pharmacokinetic data by this group (1) first demonstrated auto-induction of GEM metabolism when patients were repeatedly GEM administered. The auto-induction effect occurred between the first and second week of GEM administration. The present study was designed to answer the following questions related to the induction of GEM-TP accumulation: (i) Does induction of GEM-TP accumulation occur in a cell line model repeatedly incubated with GEM? (ii) Is the mechanism of increased accumulation via an increase in dCK mRNA expression? (iii) Is the induction specific to GEM?

Under the conditions examined, a 10-fold induction of GEM-TP accumulation was observed with repeated exposure of the HCT116 cell line. The conditions examined utilised clinically relevant concentrations and were associated with low levels of cytotoxicity (<10% cell death, data not shown). This therefore provides a preclinical model that is comparable to the in vivo situation.

The induction of GEM accumulation was also seen when the HCT116 cells were pre-incubated with another nucleoside analogue (Ara-C), non-nucleoside DNA-damaging agents (cisplatin) but not with a control nucleoside analogue (5’-azaC) that is associated with DNA methylation rather than direct DNA damage. This demonstrates that this effect is not specific to GEM and argues strongly that it is a generalised response to either DNA damage or inhibition of DNA synthesis.

It is of interest that the degree of induction was similar between GEM, Ara-C and cisplatin but was less with oxaliplatin. This was despite observing similar degrees of cytotoxicity for cisplatin and oxaliplatin at the concentrations studied. The difference is not due to differences in signalling using the MMR pathway as this pathway is deficient in HCT116 cells. The reason for this observation is therefore unexplained and is worthy of further study. Such a finding is potentially relevant for substitution of cisplatin for oxaliplatin in clinical GEM combinations.

This work is consistent with that of other investigators also have demonstrated increased intracellular uptake of GEM with cisplatin (11). Previous studies have demonstrated an ability of DNA-inhibiting agents to increase dCK activity (4). Increases in dCK activity have also been seen with high concentrations of 5’-azaC (6) and agents inhibiting DNA synthesis without DNA damage (2). This model was therefore used to investigate whether the induction of GEM observed occurred because of an increase in dCK mRNA expression. It was shown that the increase in GEM accumulation seen was not due to an increase in dCK mRNA. This work contrasts with two independent studies.
(13, 14) that demonstrated the ability of GEM to increased dCK mRNA transcripts in various cell line models. These differences may be cell line- or concentration-dependent. There are multiple potential mechanisms for the observed increase in GEM-TP. One potential mechanism for increasing dCK activity and GEM accumulation is the post-translational modification of dCK. The activity of dCK has been shown to be up-regulated post-translationally through phosphorylation at a specific amino-acid residue (Ser74) (15, 16). A further potential mechanism for auto-induction is changes in GEM transport, nucleotide depletion mediated by ribonucleotide reductase inhibition (17), or a reduction in deamination. These remain possibilities for further exploration.

In conclusion, this study has shown that the induction of GEM-TP as observed in the clinical trial could be replicated in a cell line model. The mechanism of auto-induction of GEM metabolism was independent of dCK mRNA expression but is likely to have been caused by an increase in dCK activity initiated by post-translational modification of the protein or by changes in transport or inactivation. This study suggests that the increase in dCK activity is a generic response when DNA synthesis is inhibited. Potential differences in inducing GEM accumulation between platinum analogues are worthy of further study.

References


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