Antiproliferative effects of clofarabine on AGS gastric adenocarcinoma cell line

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ABSTRACT

\textbf{Background:} Stomach cancer is the third leading cause of cancer deaths worldwide. Clofarabine as a new nucleoside analogue has antiproliferative effect on different cancer cells. In this study the antiproliferative effects of clofarabine on human stomach adenocarcinoma cell line, AGS was evaluated.

\textbf{Materials and methods:} The induction of cell death and stimulation of apoptosis by clofarabine was studied on AGS cells using MTT assay and flow-cytometry.

\textbf{Results:} Incubation of AGS cells with clofarabine for 24h, 48h and 72h induced IC50 of 0.184\textmu M, 0.919\textmu M and 1.652\textmu M, respectively. Also early apoptosis occurred in 43.10\% of clofarabine-treated AGS cells (in 24h). In addition, more incubation times yielded less percentages of early apoptotic cells, 27.20\% and 20.80\% for 48h and 72h, respectively.

\textbf{Conclusion:} Our findings suggest that clofarabine has dose-dependent cytotoxic effects on AGS. Cells are more sensitive to drug in shorter incubation times, probably due to elimination of compound after 24h.

\textbf{Key words:} AGS, chemotherapy, flow cytometry, MTT assay, Gastric cancer
INTRODUCTION

Stomach cancer, also called gastric cancer, is the third leading cause of cancer deaths worldwide due to its poor prognosis \(^1\)-\(^3\).

It is estimated that about 24,000 new cases of stomach cancer will be diagnosed and more than 10,000 deaths will occur from this type of cancer in the United States in 2015 \(^4\).

Different risk factors have been described for stomach cancer, including: gender, age, ethnicity, geography, *Helicobacter pylori* infection, stomach lymphoma, diet, tobacco use, overweight or obesity, previous stomach surgery, pernicious anemia, hypertrophic gastropathy, blood type A and inherited cancer syndromes \(^4\).

There are different types of stomach cancer including adenocarcinoma, lymphoma, gastrointestinal stromal tumor (GIST) and carcinoid tumor \(^5\).

The main strategies which are currently applied for stomach cancer treatment are surgery, targeted-, radiation-, immuno- and chemo-therapy \(^6\).

Chemotherapy is considered as the standard method for early treatment of metastatic stomach cancer because in this situation surgery is not beneficial \(^7\).

Chemotherapeutic drugs can be given to patients prior or following surgery which are called neoadjuvant and adjuvant treatment, respectively \(^7\).

There are some common chemotherapeutic drugs for treatment of stomach cancer, including 5-FU (fluorouracil), capecitabine, carboplatin, cisplatin, docetaxel, epirubicin, irinotecan, Oxaliplatin and Paclitaxel \(^8\).

All these drugs have different mechanisms of action and may be used alone or in combination with other chemotherapeutic or targeted drugs depending on the stage of the cancer and other reasons. However, side effects of these drugs reduced their permanent usage \(^7\).

Additionally, efficacy of chemotherapeutic drugs has been reduced owing to various drug resistance mechanisms. Therefore, more investigations are needed to perform for designing successful drugs \(^10\).

One ideal anti-cancer drug must be able to induce apoptosis in cancer cells without affecting normal ones \(^11\).

Blebbing, cell shrinkage, chromosomal DNA fragmentation, chromatin condensation and accumulation of phosphatidylserine (PS) on the cell surface are some typical features of apoptotic cells \(^6\).

New studies are in progress to test new anti-cancer drugs with enhanced efficacy and fewer side effects \(^12\).

Nucleoside analogues are a group of anti-cancer drugs. Owing to their structural similarity to physiological nucleosides, they can be taken up by cells, metabolized, and incorporated into DNA or RNA and thus inhibit DNA or RNA synthesis. These types of drugs need to be activated by phosphorylation in order to gain their action as cytotoxic agents \(^13\).

Fludarabine, cladribine, pentostatin, gemcitabine, capecitabine, nelarabine, decitabine and clofarabine are some of these FDA-approved nucleoside analogues used in the treatment of cancers \(^8\).

Clofarabine is a deoxyadenosine analogue approved by FDA for treating pediatric ALL (Acute Lymphoblastic Leukemia) \(^14\)-\(^16\).

Studies have demonstrated that clofarabine has a strong *in vitro* growth inhibitory and cytotoxic activity on different types of tumor cell lines such as non-small-cell lung, colon, central nervous system, melanoma, ovarian, renal, prostate and breast \(^13\),\(^17\).

In spite of these results, the effects of clofarabine on the stomach cancer is unknown and there is no report regarding the effects of clofarabine on the stomach cancer cell lines.

In the present study, we examined the effects of clofarabine on the *in vitro* proliferation of...
AGS (Stomach Adenocarcinoma Cell line) cells, originated from gastric carcinoma using MTT assay. In addition, apoptosis of the cells were detected using flow cytometry method.

**Materials & Methods**

**Materials**

Sorenson’s glycine buffer (0.1M glycine, 0.1M NaCl optimized to pH: 10.5 with 1M NaOH) was purchased from Merck Co, Germany.

AGS (ATCC® CRL1739™) Human gastric adenocarcinoma epithelial cell line (code: C131) was obtained from National cell bank of Iran (Pasteur Institute, Iran).

FBS (Fetal Bovine Serum) (Catalog number: 16000-044), trypsin-EDTA (Catalog number: 25200-056) and RPMI-1640 (Roswell Park Memorial Institute medium-1640) (Catalog number: 31800-022) were from Gibco, Invitrogen (UK).

MTT (3-(4,5-Dimethyl-2-thiazoly)-2,5-diphenyl-2H-tetrazolium bromide) (M5655), PBS (Phosphate Buffered Saline) (0.2 g/L KCl, 0.2 g/L KH₂PO₄, 8 g/L NaCl, and 1.15 g/L Na₂HPO₄), Trypan blue (T6146), streptomycin (S9137), penicillin G (P3032), DMSO (Dimethyl Sulfoxide) (D2650), Sodium bicarbonate (S5761) and clofarabine (C7495) with Molecular Weight 303.68, were all obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA).

Annexin V Apoptosis Detection Kit FITC (Catalog Number: 88-8005) was obtained from eBioscience, Inc., USA.

**Methods**

**Cell culture**

For this experimental study, AGS stomach cancer cell line was grown in a monolayer culture in 25 cm² T flasks in RPMI-1640 medium supplemented with 10% heat inactivated FBS, Penicillin G (≥1477 units per mg, 80 mg/L, 100 I.U. /mL), Streptomycin (60 μg/mL) and NaHCO₃ (2 mg/mL). Cells were maintained in a humidified cell culture CO₂ incubator INCOmed (Memmert GmbH + Co.KG, Germany) at 37°C and 5% CO₂.

**Cell count calibration**

To optimize the number of cells to be seeded in each well of 96 well plate for cell viability tests, a wide range of cell densities were cultured in tetraplicate manner. Different concentration of cells in complete RPMI-1640 medium, from 500 cells/well to 28000 cells/well, were seeded in a 96-well cell culture plate. Then, a standard MTT assay process was conducted. The cell density which yielded an absorbance between 0.75 and 1.25 was considered as the best cell number.

**Cell viability and MTT-based cytotoxicity test**

Cells in the exponential phase of growth were exposed to different concentrations of clofarabine. Cytotoxic effects of clofarabine was studied using MTT assay after 24, 48 and 72 h incubation of the cells with clofarabine.

A sub-confluent monolayer culture was trypsinized and then, cells were collected in a complete growth medium. The cell suspension was precipitated by centrifugation (200g, 5 min, and 4°C) and the pellet was then used for cell count. 10,000 cells/well were cultivated in a flat base 96-well cell culture test plate. After 24 h of incubation, cells were treated with different concentrations (0.059-40μM) of clofarabine for 24, 48 and 72 h in the quadruplicate manner. After each period of incubation, medium was removed and the cells were fed with 200 μL of fresh medium and 50μL of 2 mg/ml MTT powder dissolved in PBS. Plates were then covered with aluminum foil and incubated for additional 4 h. In the next step, wells content was removed and was replaced with 200 μL pure DMSO and 25μL Sorensen’s glycine buffer. Finally, the absorbance measurement was determined at 570 nm using the ELx808™ Absorbance Microplate Reader (BioTek Instruments, Inc., USA.) (With a reference wavelength of 630 nm). The Drug concentrations producing survival just above and below the 50% level were used in a linear regression analysis to calculate the IC50 (The half maximal inhibitory concentration).
Flow cytometry

Cells in exponential phase of growth were trypsinized and, 500,000 cells/ well were cultured in a 6-well cell culture test plate. After 24h of incubation, the IC50 concentration of drug was added to each well and cells were treated with drug for 24, 48 and 72h. Harvested cells were centrifuged using a Refrigerated Centrifuge (Spin control Professional No. 10349, SIGMA Laborzentrifugen GmbH) to evaluate apoptotic effects on stomach adenocarcinoma cells (AGS) using the Annexin V apoptosis detection kit FITC (eBioscience, Inc., USA). Cells were resuspended in 1X binding buffer at 1-5×10^6 cells/ml and 5μL of fluorochrome-conjugated Annexin V was added to 100 μL of the cell suspension and incubated for 15 minutes at room temperature. After that, Propidium Iodide Staining Solution (cat. 00-6990, eBioscience, Inc., USA) was added to the cell suspension. Finally, stained samples were analyzed by BD FACSCalibur Flow Cytometry System (BD Biosciences, CA, USA).

Statistical analysis

Statistical comparisons were made by Student’s t test. All statistical analyses were performed using SPSS software version 16.0. P<0.05 was considered as statistically significant. All graphs were drawn applying Microsoft Excel version 2013 and GraphPad PRISM version 6 and SigmaPlot 11.0.

Flow cytometry data analysis was performed using FlowJo vX 10.0.7r2 (Tree Star, Inc., Ashland OR).

Results

Cell Count Calibration

To calibrate the optimal cell density to be cultured in each well, an experiment was conducted. (Results are shown in figure1)

MTT assay was conducted to assess the IC50 value of clofarabine induced cell death in AGS cells for 24h. (Figure 2)

IC50 value for 48h was assessed by MTT assay. (Figure 3)

MTT assay was conducted to assess the IC50 value in 72h. (Figure 4)

AGS cells were incubated with clofarabine for 24, 48 and 72h. Apoptosis increased in AGS cells after 24 h. However, apoptosis reduced gradually by time. (Figures 5 & 6)

Our data showed that clofarabine has a significant effect on AGS apoptosis in 24h. FACS analysis showed 43.1% early apoptosis and 5.72% late apoptosis in 24h. (Figure 6)

Discussion

Clofarabine is a purine nucleoside analogue which shows resistance to phosphorolytic cleavage and deamination. This compound was made in 1983 and developed by Southern Research Institute to overcome the limitation of fludarabine and cladribine in some patients with hematological malignancies. It was approved by FDA in 2004 for treatment of pediatric leukemia.

The primary antitumor activity of clofarabine is performed by DNA synthesis inhibition. It inhibits RNA and protein synthesis only at high concentrations. Clofarabine is phosphorylated via deoxycytidine kinase. Clofarabine triphosphate (Clofarabine-TP) inhibits ribonucleotide reductase which is a critical enzyme involved in the de novo synthesis of deoxynucleotides.

Given data suggest that accumulation of Clofarabine-TP in whole cells, was most associated with specific inhibition of DNA synthesis.

Clofarabine-TP can efficiently substitute for normal nucleotides in apoptosome functions.

The inhibition of DNA synthesis by Clofarabine is responsible for the induction of the apoptotic response in replicating cells.

Waud et al. in 2000 evaluated the cytotoxic effects of clofarabine in nine human cell lines. As a result of their work, clofarabine has an
anticancer activity in a wide variety of leukemia and solid tumor cell lines with a broad range (0.028 to 0.29 μM)\textsuperscript{23}.

National Cancer Institute tested clofarabine on 60 human cancer cell lines in their developmental program panel and found that clofarabine potently inhibits growth activity on 35 out of 60 cell lines (GI50 values = <0.0001–0.45 μM). The tumor cell types sensitive to clofarabine included non-small-cell lung, colon, central nervous system, melanoma, ovarian, renal, prostate and breast cells\textsuperscript{13}.

To best of our knowledge, this study for the first time assessed the cytotoxic and antiproliferative effects of clofarabine on AGS stomach cancer cell line. Our data shows that clofarabine has cytotoxic effects on AGS human gastric adenocarcinoma cell line and its cytotoxic effect is dose dependent.

The calculated IC50 value for clofarabine was 0.184, 0919, 1.62 μM for 24, 48 and 72 hours, respectively. (P<0.01)

In addition, our data suggest that clofarabine has anti-proliferative activity in micro-molar doses and its effects are stronger in shorter incubation time. The reason might be the degradation or elimination of the drug after 48 and 72 hours.

**Conclusion**

In conclusion, both cytotoxic and apoptosis assays show that clofarabine has anti-proliferative and cytotoxic effects on AGS gastric adenocarcinoma cell line, and it has more effect in shorter incubation times.

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**Conflicts of interest**

The authors report no declaration of interest.

**References**


Figure 1. Cell count calibration. Optimal cell number to be seeded in each well was measured. The cell number which yielded an absorbance of 0.75 to 1.25 was the best number of cells. In this experiment, the best calculated cell number was 14,000 cell/well for 24h.

![Cell count calibration graph](image1)

Figure 2. Logarithmic-dose vs. response graph (MTT assay 24h). The calculated logIC50 for clofarabine in 24h was -6.735 and the IC50 was 1.840e-007 M. Hill Slope describes the steepness of the family of curves. A Hill Slope of 1.0 is standard. In this figure the Hill slope is 1.000. The value R² (R square) quantifies goodness of fit. It is a fraction between 0.0 and 1.0, and has no units. Higher values indicate that the model fits the data better. In this graph, R square is 0.8456 and DF=59. (P < 0.0001 and this difference is considered to be extremely statistically significant.)

![Logarithmic-dose vs. response graph](image2)
Figure 3. Logarithmic-dose vs. response graph (MTT assay 48h). The calculated logIC50 for clofarabine in 48h was -6.037 and the IC50 was 9.186e-007 M. Hill slope = 1.004; $R^2 = 0.9139$; DF = 20. ($P < 0.0001$ and this difference is considered to be extremely statistically significant.)

![MTT assay (48h)](image1)

Figure 4. Logarithmic-dose vs. response graph (MTT assay 72h). The calculated logIC50 for clofarabine in 72h was -5.782 and the IC50 was 1.652e-006 M. Hill slope = 3.054; $R^2 = 0.8858$; DF = 29. ($P = 0.0003$ and this difference is considered to be extremely statistically significant.)

![MTT assay (72h)](image2)
Figure 5. FACS plots for clofarabine effect on AGS cells after a. 24h, b. 48h, c. 72h.

Figure 6. Comparison of Flow cytometry results in different times.

References