Effects of manumycin combined with methoxyamine on apoptosis in myeloid leukemia U937 cells

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Background and Objective: Repair of DNA damage is important to cell survival. Our previous study showed DNA damage response induced by manumycin in cancer cells. We hypothesized that methoxyamine, an inhibitor of base-excision repair, can enhance the antineoplastic effect of manumycin. This study was to investigate apoptosis induced by manucycin combined with methoxyamine in myeloid leukemia cell line U937, and to explore the role of mitochondrial apoptotic pathway in apoptosis induction of the two drugs. Methods: U937 cells were treated with various concentrations of manumycin and/or methoxyamine for 48 h. The cell viability was analyzed by MTT assay. Colony formation was evaluated by soft agar clonogenic assay. Cell apoptosis was investigated by flow cytometry. Protein expressions of cytochrome c, caspase-9, and poly ADP-ribose polymerase (PARP) were determined by Western blot. Results: The dose-response curve of manumycin was shifted to the left after addition of methoxyamine. The combination index (CI) was less than 1 in U937 cells (p < 0.05), indicating a synergistic effect of manumycin and methoxyamine. Rates of colony formation of U937 cells treated with 1µmol/L manumycin, or 5 mmol/L methoxyamine, or the combination of the two were 0.3641 ± 0.0463, 0.7541 ± 0.0379, and 0.0473 ± 0.0024, respectively compared with that of control cells (p < 0.05). Moreover, the drug combination resulted in enhanced apoptosis in U937 cells. The apoptotic rates of the control, manumycin, methoxyamine and combination group were (2.34 ± 0.30)%, (8.80 ± 0.95)%, (2.21 ± 0.19)% and (13.37 ± 0.91)%, respectively. The combination of manumycin with methoxyamine also promoted the release of cytochrome c from mitochondria into the cytosol, activated caspase-9, and led to appearance of specific cleavage of PARP in U937 cells. Conclusion: Methoxyamine enhances manumycin-induced apoptosis in U937 myeloid leukemia cells.

Acute myeloid leukemia (AML) is a serious fatal disease. Although current chemotherapy and other treatment regimens have improved the remission rate and overall survival, majority of AML patients still succumb to death. Therefore, it is crucial to explore a new effective treatment regimen, in particular, an ideal combined therapy to improve efficacy in AML. Recent studies have focused on inducing apoptosis of tumor cells through intervention in signaling pathways. Farnesyltransferase inhibitors (FTIs) are a new class of signal-transduction inhibitors, which are currently under investigation for leukemia treatment. They were originally developed to block ras proto-oncogene. Ras is the protein product of ras oncogene. Mutated or abnormal ras-gene expressions are found in 25% of AML patients. After farnesylation, the precursor form of ras is located on the inner side of the cell membrane, thus to induce the mitotic signal pathway of tyrosine kinase. FTIs are able to selectively inhibit farnesyltransferase effectively. Selleriet et al. have discovered that FTIs could induce apoptosis of chronic myelogenous leukemic cells. Manumycin was first discovered in a streptococcus culture, which exerted inhibition on farnesyltransferase activity. Our previous study has found that manumycin induced apoptosis via DNA damage and DNA damage response in cancer cells. Methoxyamine disrupts base-excision repair of DNA, and it becomes a potential treatment target for restoration of chemotherapy sensitivity in tumor cells through endonuclease cleavage. Methoxyamine could enhance the antineoplastic effects of alkylating agents and radiotherapy. If base-excision repair plays an essential role in DNA repair as well as in cell survival, methoxyamine could enhance apoptosis induced by manumycin. Thus, this study was to investigate apoptosis induced by manumycin combined with methoxyamine in myeloid leukemia cell line U937.

Materials and Methods

Cell lines and reagents. Leukemia cell line U937, provided by Type Culture Collection, USA, was cultured in RPMI1640 medium containing 10% calf serum. U937 cells at their exponential phase of growth were harvested for experiment. Manumycin, methoxyamine,
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MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium), dimethyl sulphoxide (DMSO) and digitonin buffer were purchased from Sigma (St. Louis, MO, USA). Storage solution (2 mmol/L) of manumycin was prepared by mixing the regent with DMSO. Before use, manumycin was diluted with culture solution into appropriate concentrations as required, where the final concentration of DMSO was less than 0.1%. RPMI1640 medium was purchased from Life Science Technology Co., Ltd, Gaithersburg, MD, U.S.A. The anti-cytochrome c antibody was the product of Santa Cruz Biotechnologies (Santa Cruz, CA). Rabbit anti-human caspase-9, mouse anti-human PARP antibodies were purchased from PharMingen (San Diego, CA, U.S.A.).

MTT cytotoxicity assay. U937 cell suspension was inoculated on a 96-well plate at an initial density of 5,000 cells per well and incubated overnight. Manumycin (0–5 μmol/L) and methoxyamine (0–9 mmol/L) were added and incubated for 48 h. Twenty microliter of MTT reagent (3 mg/mL) was added into each well, and incubation for another four h, followed by cell centrifugation at 700 × g for 15 min. After discarding the supernatant, 200 μL of DMSO was added into each well to lyse cells. The absorbance value (A value) was recorded at 635 nm using a Tecan SpectraFluor Plus microplate reader (Tecan Inc. Switzerland).

Soft agar clonogenic assay. Cells were grown in six-well plates overnight and then treated with manumycin and methoxyamine for 48 h. The concentration of manumycin was reduced to 1 μmol/L so that the colony count would not be too low. The concentration of methoxyamine was 5 mmol/L. After drug treatments, cells were suspended in an equal volume of culture medium. The bottom agar layer was prepared using 0.5% Difco Bacto agar in small dishes. Equal volume of the cell suspension and 0.8% Difco Bacto agar in RPMI 1640 medium was added to each plate containing 0.5% soft agar. Plates were incubated at 37°C for 14 days and stained with 0.5 ml of 0.005% crystal violet. The number of colony formation was recorded using a digital camera and analyzed with Scion Image (a version of NIH Image adapted for Windows PC).

Annexin V flow cytometry assay. Annexin V-PE was used to detect cell apoptosis. The Annexin V binding method was used to determine migration of phosphatidylserine from the inner side to the outer side of the cell membrane, which is an early response of apoptosis. U937 cells were seeded onto small dishes and incubated overnight, then treated with 2 μmol/L manumycin or 5 mmol/L methoxyamine or the two in combination for 6 h. After centrifugation, cells were collected and suspended in binding buffer containing Annexin V, analyzed by FCM.

Western blot. Preparation of cell lysates. After incubation with different treatments, U-937 cells were washed with cold PBS, lysed on ice using lysis buffer. Lysated DNA was removed using ultrasonic vibration at 4°C. Cell debris was removed after centrifugation at 20630×g. Protein concentration was determined and protein was stored at -80°C.

Cytoplasm extract. After incubation with different treatments, U-937 cells were washed with PBS twice, incubated with digestion buffer to extract cytoplasm protein. Cell debris was removed by centrifugation at 20630 × g at 4°C. The supernatant was transferred into a new tube. After the measurement of protein concentrations, protein was stored at -80°C.

SDS-PAGE and Western blot. SDS-PAGE was performed using the standard method. After electrophoresis, protein was transferred onto a PVF membrane. PBS containing 5% skimmed milk and 0.05% Tween 20 (V/V) was used to block non-specific proteins. Subsequently, the membrane was incubated with the primary antibody and then with the HRP-conjugated secondary antibody for 60 min. After PBST rinse for three times, the membrane was incubated with ECL solution (Amersham Pharmacia Biotech Piscataway) for 1 min. Finally, images were developed and recorded on Kodak X-AR films.

Results

Effects of methoxyamine on manumycin-induced cytotoxicity in U937 cells. Methoxyamine enhanced manumycin-induced cytotoxicity in U937 cells at a dose-dependant manner. As shown in Figure 1, the dose-response curve of manumycin and methoxyamine was obtained. The combination index (CI) of less than one indicates a synergistic effect, CI greater than one indicates an antagonistic effect, and CI equals to one indicates an additive effect. CI values were calculated using CalcuSyn from BioSoft (Furguson, MO) computer software.

Effects of methoxyamine on manumycin-induced inhibition of clonogenicity in U937 cells. After 14 days of inoculation, a large number of colonies were formed in control group, slightly less in methoxyamine group, dramatically less in manumycin group, and almost none in drug combination group (Fig. 2). The relative rates of colony formation in manumycin group, methoxyamine group, and the combination group were 0.3641 ± 0.0463, 0.7541 ± 0.0379, and 0.0473 ± 0.0024 respectively, as compared to that of the control group (p < 0.05).

Effects of methoxyamine on manumycin-induced apoptosis in U937 cells. Manumycin significantly induced apoptosis in U937 cells (p < 0.05), but methoxyamine had no effect on apoptosis. The combination of manumycin and methoxyamine resulted in enhanced apoptosis in U937 cells compared with manumycin alone (p < 0.05).
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Effects of methoxyamine on manumycin-induced activation of mitochondrial apoptotic pathway in U937 cells. The combination of manumycin and methoxyamine enhanced activation of caspase-9, decreased procaspase-9, induced appearance of specific cleavage of caspase-9 in U937 cells. Meanwhile, the combination of manumycin and methoxyamine promoted the release of cytochrome c from mitochondria into the cytosol and increased specific cleavage of PARP (Fig. 4)

Discussion

Our previous studies have shown that manumycin could cause DNA damage and induce DNA damage response. The interaction between manumycin and methoxyamine inhibits DNA damage repair. In this study, we found that methoxyamine enhanced the cytotoxic effect of manumycin by enhancing apoptosis.

Apoptosis is a cell death process activated by intrinsic DNA endonuclease. It is mediated by cell surface receptors or the mitochondrial pathway, which interact with each other and enhance the death signal. Mitochondrial pathway is the major apoptotic pathway and it plays a critical role in cell apoptosis. Therefore mitochondria are the major integration sites of many proapoptotic and antiapoptotic signals. Mitochondrial proteins, such as cytochrome c and SMAC are important in apoptosis regulation. Cancer cells can be killed through cell apoptosis, inhibition of proliferation, and DNA damage. Among them, induction of apoptosis is the most effective strategy in cancer therapy. We found that manumycin and methoxyamine caused release of cytochrome c from mitochondria to cytosol, markedly activated caspase-9 and PARP cleavage. Our results have provided evidences to support the fact that methoxyamine enhances manumycin-induced apoptosis in U937 cells through mitochondrial apoptotic pathway.

Base-excision repair of DNA is a potential therapeutic target to increase antineoplastic effects of chemotherapy. Our previous study has shown that manumycin damages DNA via reactive oxygen species. Methoxyamine enhances manumycin-induced apoptosis

![Figure 2: Effects of manumycin (Manu) combined with methoxyamine (MX) on colony formation in U937 cells](image)

![Figure 3: Effect of methoxyamine on manumycin-induced apoptosis in U937 cells](image)

The apoptotic rates of the control, manumycin, methoxyamine, and combination group were 2.34 ± 0.30%, 8.80 ± 0.95%, 2.21 ± 0.19%, 13.37 ± 0.91%, respectively. The rate was significantly higher in the combination group than the control, manumycin, and methoxyamine groups (p < 0.05) (Fig. 3A–D).
through blocking DNA base-excision repair and preventing endo-nuclease cleavage. To assess the overall antineoplastic effect of the combination of methoxyamine and manumycin, we measured both cell survival and clonogenicity on soft agar and found that methoxyamine enhanced manumycin-induced inhibition of clonogenicity and survival. This suggests the importance of base-excision repair in the survival of manumycin-treated cells. Hence, manumycin plus methoxyamine is a rational treatment regimen against AML.

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References