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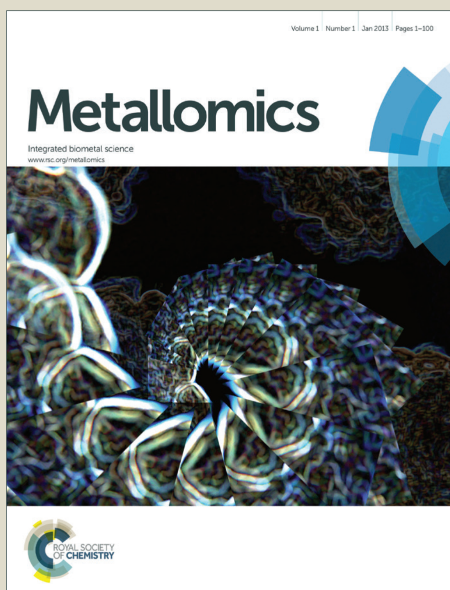

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Combination of Arsenic and Cryptotanshinone Induces Apoptosis through Induction of Endoplasmic Reticulum Stress-Reactive Oxygen Species in Breast Cancer Cells

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Abstract

Arsenic trioxide has been successfully used for the treatment of patients with acute promyelocytic leukemia (APL) worldwide. Recently, it has also been further developed to treat the solid tumors in clinical trials. However, the therapeutic effects on malignant tumors appeared to be unsatisfactory, as these cells exhibited resistance towards arsenic. In this study, we explored new therapeutic strategies for treatment of human breast cancer MCF-7 cells based on arsenic metabolites. The MCF-7 cells were exposed to the three arsenic species, namely, inorganic arsenite (iAs^{III}) and its intermediate metabolites monomethylarsonous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III}) alone or in combination with cryptotanshinone (CPT) to establish their anticancer effects against MCF-7 cells. Surprisingly, MCF-7 cells showed to be resistant to both iAs^{III} and CPT alone, however, showed to be relative sensitive to treatment when exposed to MMA^{III} and DMA^{III} alone. Conversely, the combination of MMA^{III} with CPT showed significantly enhanced anticancer effects on MCF-7 cells at low doses, but no appreciable effect was observed on exposure to other two arsenic species with CPT. In addition, remarkable redistribution of pro-apoptosis related proteins Bax and Bak were observed in mitochondria, together with activation of Poly (ADP-ribose) polymerase (PARP) and caspase-9 after exposure to combination of MMA^{III} with CPT. Furthermore, we clearly found that induction of apoptosis in MCF-7 cells was predominantly triggered by endoplasmic reticulum (ER) stress after exposure to the combination of MMA^{III} with CPT.

Keywords: arsenic trioxide, dimethylarsinous acid, monomethylarsonous acid, cryptotanshinone, human breast cancer, ER stress

Abbreviations: APL, acute promyelocytic leukemia; As_2O_3, arsenic trioxide; iAs^{III}, arsenite; CPT, cryptotanshinone

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Introduction

Arsenic trioxide (As_2O_3) is widely used for the treatment of patients with acute promyelocytic leukemia (APL) and has showed remarkable clinical success.[1] Thereby, it has also been used to treatment of other forms of tumors and diseases.[2,3] However, many reports have indicated that As_2O_3 as a single-agent has not been as effective as anticipated against non-promyelocytic leukemia and other malignant tumors in clinical trials.[4,5]

Generally, arsenic trioxide is commonly hydrolyzed to arsenite (i.e., $As_2O_3 \rightarrow iAs^{III}$) and then metabolized in liver to trivalent monomethylarsonous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III}) species by arsenic methyltransferase (AS3MT) in the body and is finally excreted into urine as low toxic pentavalent methylated forms.[6-8] Moreover, these trivalent intermediate metabolites have shown to be more cytotoxic towards different cell lines as compared to their precursor; iAs^{III}.[9,10] However, so far there is no information regarding the anticancer effects of arsenic intermediate metabolites (i.e., MMA^{III} and DMA^{III}) in clinic trials. Thus, its arsenic intermediate metabolites may have more strong anticancer effects on malignant cancers than that of inorganic iAs^{III}.

To date, many scientists have tried to develop the single-agent As_2O_3 for treatment of solid tumors in clinic trails, but it has not shown any significant responses against solid cancer including hepatocellular carcinoma (HCC) or multiple myeloma (MM).[11] Although, arsenic trioxide has showed potent antiproliferative and proapoptotic effects in pancreatic cancer cells, however, despite of the promising *in vitro* data, As_2O_3 has not demonstrated any satisfactory outcomes in pancreatic cancer patients.[4] Moreover, Hayashi et al.[12] has found that As_2O_3 around 2~5μM could induce apoptosis in drug-resistant multiple myeloma cell lines or primary patient cells, but the exposure concentrations is indeed very high. Such reports suggest that the efficacy of As_2O_3 as a single-agent is not significantly acceptable for the treatment of non-APL cancer. As an alternate, efforts may be made to reduce the dose of arsenic trioxide or may be combined with other standard regimen in reversing the chemo resistance.

Recently, a great increase in the public concern and awareness about breast cancer has been observed. Human breast adenocarcinoma cell line MCF-7, an estrogen receptor (ER) positive breast cancer cell line, has been used as a cellular model to study As_2O_3 treatment. However, clinically achievable concentrations of As_2O_3 at 2μM (i.e., 4μM iAs^{III}) have shown no effectiveness on breast cancer in clinic trials.[12,13] Differently from the singe-agent treatment, Baumgartnerwe et al., has reported that the combination of As_2O_3 (1μM) with docosahexaenoic acid (DHA) could remarkably enhance the apoptotic effect in drug-resistant leukemia cells as compared to As_2O_3 treatment alone. Moreover, they observed that the combination

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treatment was also effective against other cancer cells that were derived from various hematologic malignancies,[14] indicating that combination of As_2O_3 with other agents as an alternative anticancer treatment strategy. Our previous work have found the combination of iAs^{III} with cryptotanshinone (CPT), isolated from the root of salvia miltiorrhiza, at sub-toxic concentration has significantly increased the anticancer effects of arsenic on human multiple myeloma (MM) cells lines as compared with the arsenic treatment alone, implying the combination of arsenic with other natural chemical compounds could improve the treatment for drug resistant malignant cancers.[15] CPT has been identified to have anti-inflammatory, anti-cancer, antioxidative and anti-angiogenic activities.[16] Especially, recent studies have indicated that CPT exerts potential anticancer activity through targeting STAT3 signaling, inhibiting the signaling pathway of the mammalian target of rapamycin (mTOR) and mTOR-mediated cyclin D1 expression and Rb phosphorylation.

Based on arsenic metabolism in body which results in the formation of active arsenic intermediate metabolites, we have tried to develop a novel therapeutic strategy for human breast cancers. Here we have examined the anti-cancer effect of arsenite and its intermediate metabolites (i.e., MMA^{III} and DMA^{III}) alone and in combination with CPT in human breast cancer MCF-7cell line. We found that the MCF-7 were much more resistant to iAs^{III} as compared to the two methylated arsenic species, however, the combination of 1μM MMA^{III} with 15μM CPT at sub-toxic concentrations was found to significantly reduce the cell survival. In addition, we found that combination of MMA^{III} with CPT induced apoptosis in MCF-7 cells predominantly through activation of ER stress, but not by STAT3 pathway. Hence, our findings suggest that combination of arsenic intermediate metabolites with CPT have much potent anticancer activity as compared to iAs^{III}, thereby this approach might be used for the treatment of human breast cancer in the clinical trials in near future.

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Material and Methods

Reagents

All reagents were of analytical grade. Milli-Q water (Millipore) was used throughout the experiment. Trizma® HCl and Trizma® Base were purchased from Sigma (St. Louis, MO, USA). L-cysteine, sodium arsenite (iAs^{III}), sodium arsenate (iAs^{V}), and dimethylarsinic acid [(CH3)2AsO(OH)] (DMA^{V}) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Monomethylarsonic acid (MMA^{V}) was obtained from Tri Chemicals (Yamanashi, Japan).

Cell culture

Human breast cancer MCF-7 cells were purchased from Chinese Academy of Sciences in Shanghai. Cells were cultured in logarithmic growth phase using RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Grand Island, USA), 100U/mL penicillin, and 100 μg/mL streptomycin, at 37°C in 5% CO_2 atmosphere. After twenty-four hours of seeding, the cultures were washed twice with PBS, fresh medium was added, and then the cells were treated with indicated doses of iAs^{III}, MMA^{III}, DMA^{III} alone or combined with CPT for 24h. In the current study, iAs^{III} will represent As_2O_3.

Preparation of Monomethylarsonous Acid (MMA^{III}) and Dimethylarsinous Acid (DMA^{III})

MMA^{III} and DMA^{III} were prepared by reducing MMA^{V} and DMA^{V} respectively with 5 molar equivalents of L-cysteine in distilled water at 90 °C for 1h.[10] The trivalent forms were confirmed by comparison of the respective retention times on a GS 220 gel filtration column by HPLC−ICP MS with those prepared from their iodide forms in distilled water under nitrogen atmosphere. Purity of MMA^{III} (98%, with 2% of MMA^{V}) and DMA^{III} (95%, and with 5% of DMA^{V}) was confirmed by HPLC-ICP MS and then were used for further experimental purpose.

MTT assay for cell viability

Human breast cancer MCF-7 cells were seeded at a density of 2×10^4 cells/100 μL/well in 96-well microtiter plates (Promega Corporation). Twenty-four hours post-seeding, the cultures were washed twice with PBS and then exposed to various concentrations of

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iAs^{III}, MMA^{III} and DMA^{III} or CPT for 24h. Then, 20µL of an MTT solution was added to each well (at the final concentration of 0.5mg/mL), and the plates were incubated for an additional 3h at 37ºC. Afterward, cell cultures were washed with PBS, and 150 µL of DMSO was added to each well. Cell viability was measured as absorbance at 570 nm with a microplate reader and the results were expressed as percentage of the control level.

Western blot analysis

MCF-7 cells were washed twice with cold D-hanks solution, followed by the whole cells using RIPA lysis buffer containing 50mM Tris-HCl, pH 7.5, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.2 mMPMSF, and a complete mini protease inhibitor tablet. Lysates were incubated on ice for 30 min and centrifuged for 30 min at 13,000×*g* at 4ºC to obtain the supernatant. Protein concentrations were determined by Bio-Rad microprotein assay using bovine serum albumin as standard. Twenty-five microgram of each protein sample was resolved by 10 or 12% SDS-PAGE and electro-blotted onto nitrocellulose membranes (Bio-Rad, Mississauga, ON). The membranes were blocked for 1h at room temperature using 5% skim milk plus 0.1% Tween-20 (PBST) and incubated overnight at 4ºC with different primary antibodies, followed by incubation with HRP-linked secondary antibodies for 1h at room temperature then after washing the proteins were visualized by enhanced chemiluminescence (ECL).

Assessment of cellular apoptosis

Cellular apoptosis was measured by Annexin V-FITC and propidium iodide (PI) staining. MCF-7 cells were treated with 1µM of iAs^{III}, MMA^{III} and DMA^{III} alone or in combination with a 15µM CPT for 24h. Later the cells were washed with PBS and re-suspended (1×10^{6} /mL). Cells were then stained with 5µL Annexin V-FITC and were incubated for 15min in dark at 37ºC. Afterward, PI (20µg/mL) was added and the samples were immediately analyzed on flow cytometer (Beckmancoulter).

Measurement of intracellular ROS by flow cytometry

The oxidation-sensitive fluorescent probe (DCFH-DA) was used to detect the intracellular ROS level, as described in our previous work.[10] Briefly, MCF-7 cells were

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treated with 1μM of MMA^{III} alone or in combination with 15μM CPT for 12h. After incubation, cells were washed with PBS and then incubated with 10μM/L DCFH-DA at 37ºC for 20 min. Fluorescence was detected by flow cytometer (Beckmancoulter).

Immunofluorescence microscopy

MCF-7 cells (1×10^5) were cultured on Chamber slides and then exposed to 1μM of MMA^{III} alone or with CPT for 12h. After washing with PBS twice, the slides were fixed by paraformaldehyde (PFA) for 30min. Cells nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, blue) for 5 min at 4 °C. After washing with PBS, phycoerythrin-conjugated streptavidin was added for 30 min before washing, mounting in Aquamount, and examination under a Zeiss (Göttingen, Germany) 510 confocal microscope. Confocal scanning parameters were set up so that the cells in the well without the compounds had no fluorescent signal.

Statistical analysis

Each viability value represents the mean±S.D. from four determinations, and IC_{50} values were calculated from the log-log plot between the percentages of viable cells. Subsequently, each experiment was performed at least three times. Statistical analysis of data was carried out using a one-way ANOVA followed by Holm-Sidak pairwise multiple comparison test (Sigmaplot, Systat Software Inc), and a probability value of less than 0.05 (*$p<0.05$) was accepted as a significant difference.

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Results

Effect of three arsenic species with or without CPT on cell viability of MCF-7 cells

Arsenic trioxide is commonly metabolized in body to mono- and di-methylated arsenic metabolites such as monomethylarsonous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III}), whereas, iAs^{III} represents As_2O_3 in the present study.

MCF-7 cells survival was determined by MTT assay after exposure to iAs^{III}, MMA^{III}, DMA^{III} and CPT alone or in combination at indicated concentration for 24h, as shown in Figure1A,B. Human breast cancer MCF-7 cells exhibited resistance to both inorganic iAs^{III} and CPT alone, whereas were found to be relatively more sensitive towards MMA^{III}, and less sensitive towards DMA^{III}. However, when the three arsenic species were given, there was no appreciable toxic effect found in MCF-7 cells following exposure to at low dose 1μM (Fig.1).

Based on these results, we further compared the cytotoxic effect of three arsenic species at 1μM with 15μM CPT on MCF-7 cells (Fig.1C-E). Interestingly, after exposure to MMA^{III} with CPT, the cells viability was significantly reduced to approximately 40% of control (Fig.1D), however, about 75~80% of cell survival was observed when MCF-7 cells were exposed to combination of iAs^{III} or DMA^{III} at 1μM with 15μM CPT (Fig.1C,E), suggesting that the combination of MMA^{III} with CPT have much stronger synergistic effect than other two species on the MCF-7 cells. In addition, the induction of apoptosis after exposure to 1μM iAs^{III}, MMA^{III} or DMA^{III} with 15μM CPT were also determined (Fig.2A). As anticipated, combination of MMA^{III} with CPT showed significant induction of cellular apoptosis (Fig.2A), and remarkable activation of poly (ADP-ribose) polymerase (PARP), the apoptosis related proteins was observed (Fig.2B). However, no appreciable effect was observed after exposure to other two species including iAs^{III} and DMA^{III} with CPT at the same conditions, which was found to be consistent with the results of MCF-7 cell viability (Fig.2B, C).

Changes in proapoptotic proteins Bax, Bak and Cyt c in cytoplasm and mitochondria of MCF-7 cells following exposure to three arsenic species with or without CPT

In order to understand the mechanism of synergistic effects for the three arsenic species with CPT were further determined in terms of induction of MCF-7 cell death, changes in proapoptotic proteins including Bax, Bak or cyt c in cytoplasm and mitochondria of MCF-7 cells, as shown in Figure3. In fact, inorganic iAs^{III} with CPT showed to slightly increase the Bax, and reduce cyt c in mitochondria (Fig.3A and a),

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3 while MMA^{III} with CPT remarkably induced the translocation of Bax from cytoplasm
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5 to mitochondria, along with increasing Bak and reducing cyt c in mitochondria
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7 (Fig.3B and b). However, no related significant changes were observed in MCF-7
8 cells after exposure to DMA^{III} with CPT (Fig.3C and c). These observations were also
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10 found to be consistent with the result of cell viability obtained after exposing MCF-7
11 cells to the combination treatment (Fig.1D), suggesting that the MMA^{III} with CPT
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13 could enhance the induction of apoptosis and has much stronger effects on MCF-7
14 cells than the other two arsenic species (i.e., iAs^{III} and DMA^{III}).
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17 *Determination of ER stress in MCF-7 cells after exposure to combination of MMA^{III}*
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19 *with CPT*
20 Phosphorylation of signal transducer and activator of transcription 3 (STAT3) can be
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22 specifically inhibited by CPT.[16] Thus, we are interested in determining the effects of
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24 the arsenic species on the inhibition of STAT3 activity. Interestingly, phosphorylation
25 of STAT3 (p-STAT3) was not observed after exposure to either of the three arsenic
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27 species alone, however, a complete inhibition of STAT3 activity was observed after
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29 exposure to the sub-toxic concentration of CPT, as shown in Figure4A. In addition,
30 we also found that CPT was able to inhibit p-STAT3 in MCF-7 cells even at low
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32 concentration; 2~10 μM (data not shown), implying that the inhibition of p-STAT3 is
33 not involved in the induction of apoptosis after exposure to the combination of arsenic
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35 with CPT. According to the above results, we found that MMA^{III} with CTP have much
36 potent effects on induction of apoptosis as compared to the other two arsenic species.
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38 Thus, we predominantly focused on the effect of MMA^{III} with CTP on MCF-7 cells in
39 subsequent experiments.
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41 It has been reported that ER stress and MAPK pathways play essential role in the
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43 regulation of cellular response such as cellular apoptosis, survival, proliferation and
44 differentiation.[17,18] In this study, we opt to find that whether the induction of apoptosis
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46 observed in MCF-7 cells after exposure to the combination of MMA^{III} with CPT was
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48 through ER stress or not, thereby ER-stress related proteins p-PERK, ATF4 and
49 CHOP or p-ASK1, p-JNK were determined at different time points (0, 1, 3, 6, 9, 12
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51 and 24h) after exposure to the indicated combination. Surprisingly, significant
52 induction of p-PERK was observed as early as 1h after exposure to the combination
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54 treatment (i.e. MMA^{III} with CPT), followed by the activation of the downstream
55 proteins; ATF4 and CHOP with the increase in the exposure time (Fig.4B). On the
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57 other hand, induction of p-ASK1 and p-JNK was also observed (Fig.4.C), suggesting
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that the induction of apoptosis by MMA^{III} with CPT mainly occurred through the ER-stress. Conversely, in this study, we were also interested in evaluating the involvement of MAPK pathway, however, we found that the MMA^{III} with CPT induced the p-erk and p-38 activation at very late time, indicating that the MAPK signaling pathway might not be the main pathway involved in the induction of apoptosis in MCF-7 cells after exposure to the combination of MMA^{III} or CPT.

Effect of p-JNK or caspase inhibitors on induction of apoptosis in MCF-7 cells after exposure to combination of MMA^{III} with CPT

Although, MMA^{III} or CPT alone could not significantly induce ER-stress in MCF-7 cells, however significant induction of the ER-stress was observed in MCF-7 cells after exposure to the combination treatment (Fig.5A). In order to further verify the involvement of ER-stress as a main pathway for the induction of apoptosis in the current study, we used two specific inhibitors to evaluate our results. Interestingly, when the cells were pretreated with p-JNK inhibitor (SP600125), the induction of apoptosis after combination treatment was significantly attenuated (Fig.5B), suggesting that MMA^{III} with CPT induced apoptosis by predominantly triggering ER-stress in MCF-7 cells. Moreover, similar results were observed after the pretreatment of caspase inhibitor (Z-VAD-FMK), as this inhibitor also prevented the induction of apoptosis by the combination treatment, indicating that the MMA^{III} with CPT induced apoptosis in MCF-7 cells was caspase dependent (Fig.5C).

Localization of reactive oxygen species (ROS) in MCF-7 cells after exposure to combination of MMA^{III} with CPT

We were further interested in determining the ER-stress-induced generation of reactive oxygen species (ROS). Thus, the generation of ROS was determined in MCF-7 cells after exposure to MMA^{III}, CPT alone or MMA^{III} with CPT in the presence or absence of antioxidant N-acetylcysteine (NAC) as shown in Figure6A. Interestingly, ROS generation was not significantly increased in cells exposed either to MMA^{III} (1μM) or CPT (15μM) alone (data not shwon), however, strikingly increased generation of ROS was observed after the exposure to the combination treatment at 12h. Moreover, this ROS generation was completely inhibited in the cells pretreated with NAC, implying that ROS also involved in the induction of apoptosis in MCF-7 cells in current study. In addition, we found that ROS was generated in both ER and mitochondria after exposure to the combination treatment, and no significant

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ROS generated in both organelles by exposure to either CPT or MMA^{III} alone (Fig.6B). Additionally, the induction of apoptosis was attenuated by pretreatment with NAC following combination treatment, indicating that the generation of ROS has involved in the apoptosis (Fig.6C).

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Discussion

Arsenic trioxide, a therapeutic agent used from thousands of years in Chinese, Mongolian and Tibetan traditional medicine, has proven to be an effective drug for the treatment of acute promyelocytic leukemia (APL).[1] This successful approach has also encouraged many scientists to use this compound for the treatment of other malignant tumors.[2-6] However, as a single-agent, As_2O_3 has shown limited efficacy against non-APL and solid tumors may be because these cells are highly resistant to As_2O_3.

An early study has reported that high dose of As_2O_3 may increase overall survival rate in multiple myeloma (MM) patients, but most of the patients may not tolerate such high-dose chemotherapy. [19] Moreover, high dose of As_2O_3 can also causes a number of adverse events like cytopenia, deep vein thromboses and infectious complications.[20] Thereby, new therapeutic approaches are needed to be developed for the use of arsenicals in clinical treatment against various arsenic-resistant tumors.[21-24]

On the other hand, it is well known that inorganic arsenic can be metabolized into mono- and dimethylated arsenic species (e.g., MMA^{III} and DMA^{III}) by arsenic methyltransferase (AS3MT) in human body.[6,7] Moreover, these two intermediate metabolites have shown to be more toxic than that of their precursor; iAs^{III} in different cells.[8-10] Thus, as an attempt to develop new therapeutic approach that can help increase the anticancer activity and reduce the toxicity of arsenicals, we have established and compared the synergistic effects of three arsenic species in combination with the natural chemical compounds (CPT) using human cancer breast cancer MCF-7 cells. The MTT assay revealed that MCF-7 cells exhibits great resistance for inorganic iAs^{III} and relative sensitivity towards the two methylated metabolites alone, in particular for MMA^{III} (Fig.1A,B). However, cell viability was significantly reduced with the sub toxic concentration of the MMA^{III} and CPT combination Fig.1D), suggesting that the CTP may probably increase the efficacy of MMA^{III} at low dose against MCF-7 cells.

Activation of mitochondrial-mediated apoptotic pathways is one of the most well characterized mechanisms of action of iAs^{III}. In previous work, we have clearly found that combination of iAs^{III} and CPT could increase the induction of apoptosis through mitochondria-mediated apoptotic pathway in human multiple myeloma U266 cells.[15] However, we could not find appreciable synergic effect of iAs^{III} with CPT in MCF-7 cells as compared to our previous work. Conversely, the combination of MMA^{III} and

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CPT has shown to have strong synergistic anticancer effect on MCF-7 cells (Fig.2). The pro-apoptotic protein Bax was markedly recruited in mitochondria followed by its reduction in cytoplasm (Fig.3), which facilitated the release of Cyt c from the mitochondria (Fig.3). As, it is well known that release of cytochrome c into the cytoplasm can lead to the formation of apoptosome (a complex of dATP, cytochrome c, Apaf-1 and procaspase-9) which results in the activation of caspase-9 that may finally induce cellular apoptosis.[25-27]

Caspase-3 has been shown to play a key role in apoptotic events such as DNA fragmentation and membrane blabbing, moreover the caspase-3 null mice have shown to die perinatally displaying hypercellularity.[28] Although it has been reported that MCF-7 cells also lack the caspase-3 proteins, the induction of apoptosis in MCF-7 cells was significantly inhibited by pretreatment of z-VAD-fmk (i.e., inhibitor of apoptosis) (Fig.5B). Correspondingly, activation of PARP and caspase9 was also clearly found in MCF-7 cells after the combination treatment, implying the apoptotic effect of MMA^{III} with CPT to be caspase-mediated (Fig.2). We further found that the induction of apoptosis occurred predominantly through the ER-stress instead of MAPK/ERK pathway (Fig.5), as ER-stress related proteins p-perk, ATF4 and CHOP or p-ASK and p-JNK were induced significantly as early as 1~3h of treatment (Fig.4). Additionally, combination treatment induced apoptosis was prevented by pretreatment with eif2 or p-JNK inhibitors (Fig.5), suggesting that the apoptosis was ER-stress mediated.

Notability, although CPT is known to be cable of inhibiting p-STAT3,[16] it seems that the combination of arsenic with CPT did not induce apoptosis through this pathway in MCF-7 cells as observed in the current study. Our previous study has demonstrated mitochondria as a target organelle for methylated induced toxicity, especially we found that MMA^{III} is capable of to inducing ROS generation in the mitochondria through inhibiting the complexes II and IV of electron transport chain (ETC).[29] However, their combination treatment was found to be much toxic to ER in the present study. Future studies need to probe the mechanism by which combination of MMA^{III} and CPT induced ROS in ER. Additionally, we also found that oxidative stress was also involved as a toxic event at late time (Fig.6A), and the localization of ROS was found to be in endoplasmic reticulum (ER) as well as in mitochondria (Fig.6B). Inhibition of ROS could attenuate the induction of apoptosis mediated by the combination of MMA^{III} with CPT (Fig.6C), suggesting that the ER-stress resulted in generation of ROS in ER which ultimately also affected the mitochondria.

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Taken together, our data demonstrate that human breast cancer is indeed resistant to the single-agent As_3O_3 treatment, but we found that combination of CPT with arsenic intermediate metabolites (particularly MMA^{III}) could strongly increase the anticancer activity even at low doses. We provided new evidence that arsenic intermediate metabolite; MMA^{III} at low concentration is able to increase the induction of apoptosis in combination with CPT, suggesting that this combination may be a good therapeutic approach for the treatment of human breast cancer.

Conflict of interest

The authors report no conflicts of interest.

Acknowledgements

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Legends to Figures

Figure1. Effect of three arsenic species with or without CPT on the viability of MCF-7 cells. Human breast cancer MCF-7 cells were exposed to indicate concentrations of iAs^{III}, MMA^{III}, DMA^{III} (**A**), cryptotanshinone (**B**) for 24h. Additionally, cells were also exposed to combination of 1μM iAs^{III} (**C**), MMA^{III} (**D**), DMA^{III} (**E**) with 15μM CPT for 24h. Cell viability was determined by MTT assay as described in Materials and Methods. Data are expressed as mean values ± S.D. Asterisks (*) indicate a significant difference from the CPT and arsenic species treated groups at $P< 0.05$.

Figure2. Determination of apoptosis in MCF-7 cells after exposure to combination of arsenicals with CPT. MCF-7 cells were exposed to 1μM concentration of iAs^{III}, MMA^{III} and DMA^{III} alone or with 15μM CPT for 24h. Induction apoptosis was determined by flow cytometry (**A**). Poly (ADP-ribose) polymerase (PARP) (**B**) and Caspase-9 (**C**) were determined by western blot. Proteins (25 μg) extracted from whole cell were separated by electrophoresis on a 12 % SDS–polyacrylamide gel as described in ‘‘Materials and methods.’’ Actin was used as a loading control. Asterisks (*) indicate a significant difference from the CPT and MMA^{III} treated groups at $P< 0.05$

Figure3. Changes in proapoptotic proteins in cytoplasm and mitochondria following exposure to combination of arsenic with CPT. MCF-7 cells were exposed to 1μM concentration of iAs^{III} (**A** and **a**), MMA^{III} (**B** and **b**) and DMA^{III} (**C** and **c**) along or with 15μM CPT for 24h. The proapoptotic proteins Bak and Bax or Cyt c in cytoplasm (**A**, **B** and **C**) and mitochondria (**a**, **b** and **c**) were determined by immunoblotting.

Figure4. Combination of MMA^{III} and CPT induces ER-stress in human breast cancer MCF-7 cells. MCF-7 cells were exposed to1μM concentration of each arsenic species alone or with 15μM CPT for 24h and determined the changes in phosphor-STAT3 (Try) protein expression (**A**). Cells were exposed to 1μM MMA^{III} with 15 μM CPT for the indicated time points to determine the changes in p-Perk, ATF4 and Chop (**B**) or p-ASK1and p-JNK (**C**) as well as p-erk and p-p38 (**D**) proteins expressions by immunoblotting.

Figure5. Effect of p-JNK or caspase inhibitors on induction of apoptosis in MCF-7 cells after exposure to combination of MMA^{III} with CPT. MCF-7 cells were exposed to 1μM MMA^{III} and 15 μM CPT alone or combination of the two compounds for 24h to determine the induction of ER-stress (**A**). The effects of

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3 pretreatment of p-JNK inhibitor (SP600125) (**B**) or caspase inhibitor (Z-VAD-FMK) (**C**) on induction of apoptosis in MCF-7 cells by combination of 1μM MMA^{III} and 15μM CPT. Cleaved caspase-3, -9 and poly (ADP-ribose) polymerase (PARP) were determined by immunoblotting using specific antibodies. Beta-actin was used as a loading control.

Figure6. Determination of Generation of ROS and localization in MCF-7 cells following exposure to combination of MMA^{III} with CPT. MCF-7 cells were exposed to 1μM concentration of MMA^{III} and 15μM CPT alone or combination of MMA^{III} and CPT for 6h to determine generation of ROS by flow cytometry (**A**). MCF-7 cells were seeded on a 6-well culture plates with cover glasses, and then cultured for 24h. Cells were pre-treated with 2μM CM-H2DCFDA (green) and ER-tracker Red (red) or Mito-tracker (red) for 30 min. After washing with PBS, cells were exposed to mentioned concentration as indicate above for 3h, (**B**). Following exposure, cells were fixed with 10% formalin, and then ROS generation was determined using a confocal laser scanning microscope. (**C**) Changes in apoptosis related proteins caspase-9 and PARP in MCF-7 cell after exposure to combination of MMA^{III} with CPT in the presence of N-acetylcysteine (NAC) for 24h. Cleaved caspase-3, -9 and poly (ADP-ribose) polymerase (PARP) were determined by immunoblotting using specific antibodies.

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Figure 1

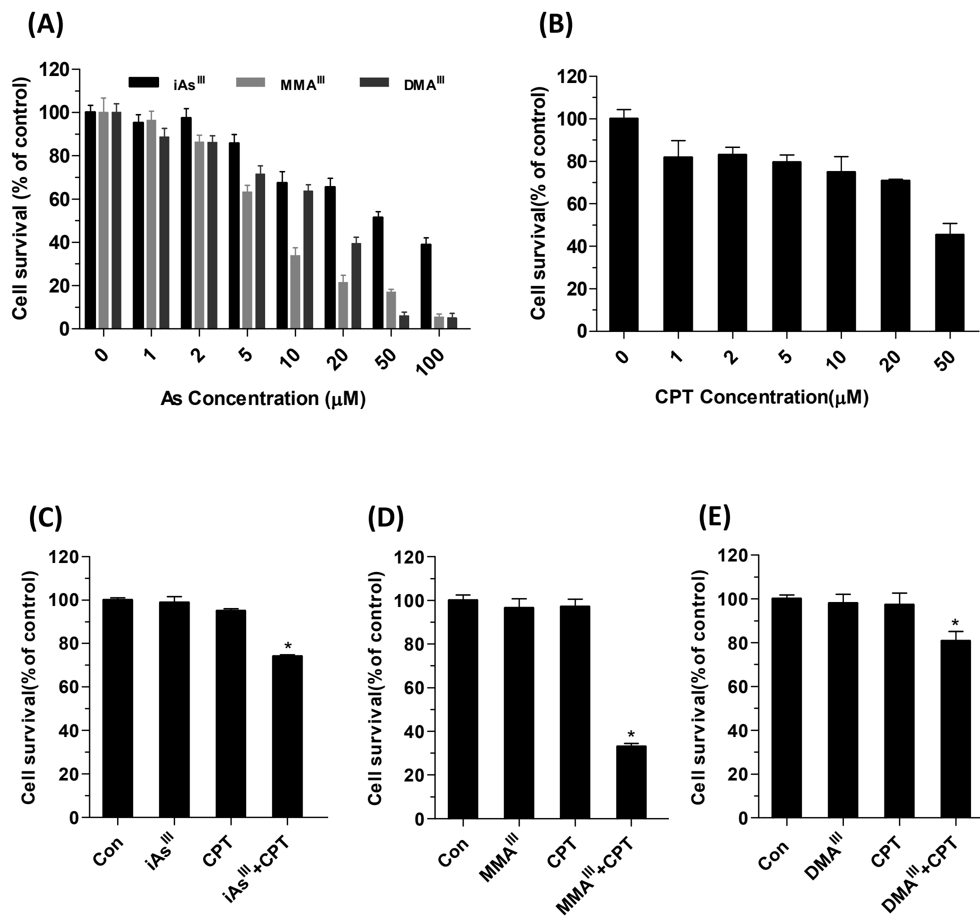

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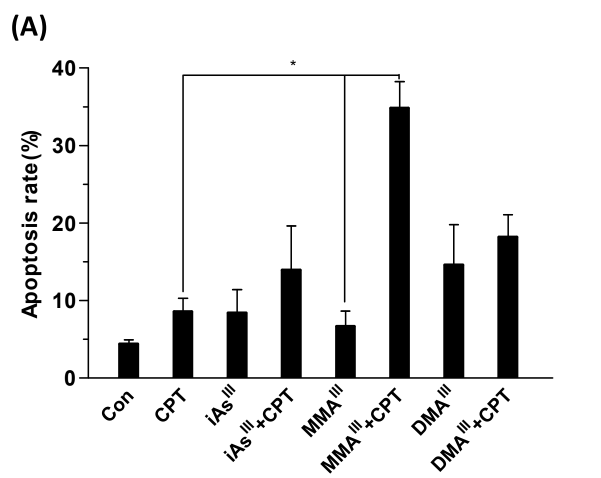
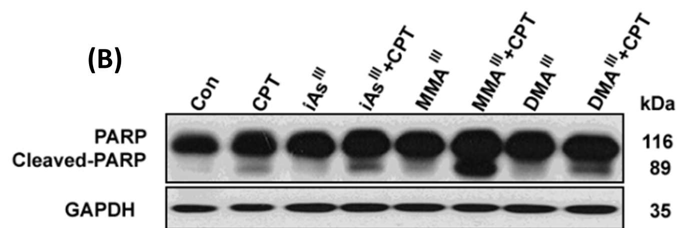
Figure 2





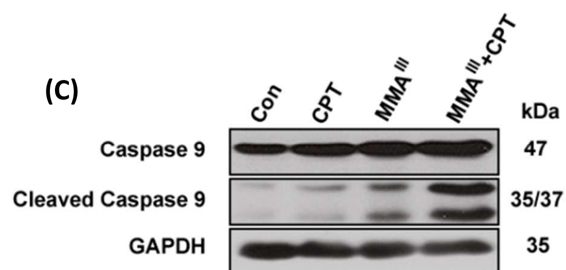


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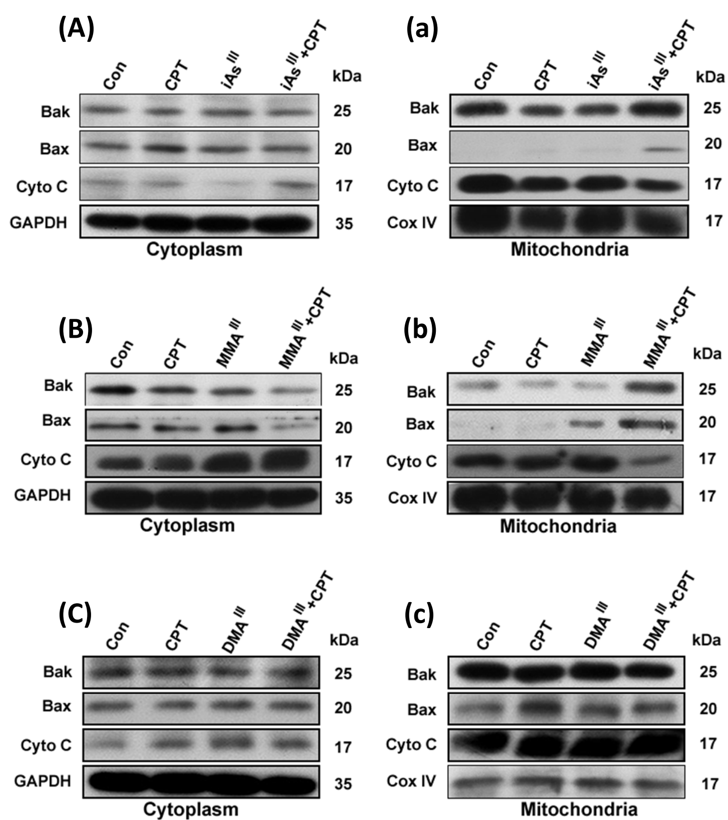
Figure 3



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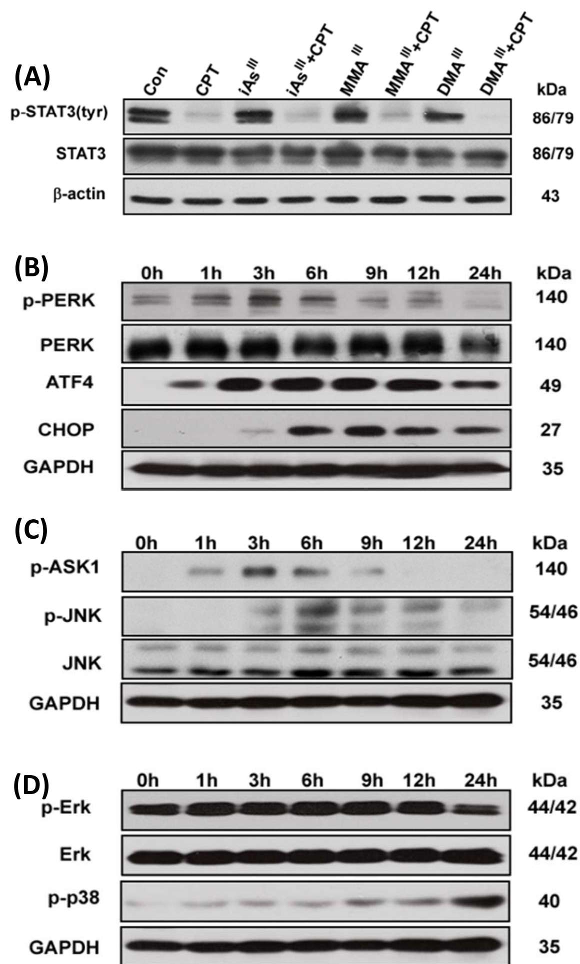

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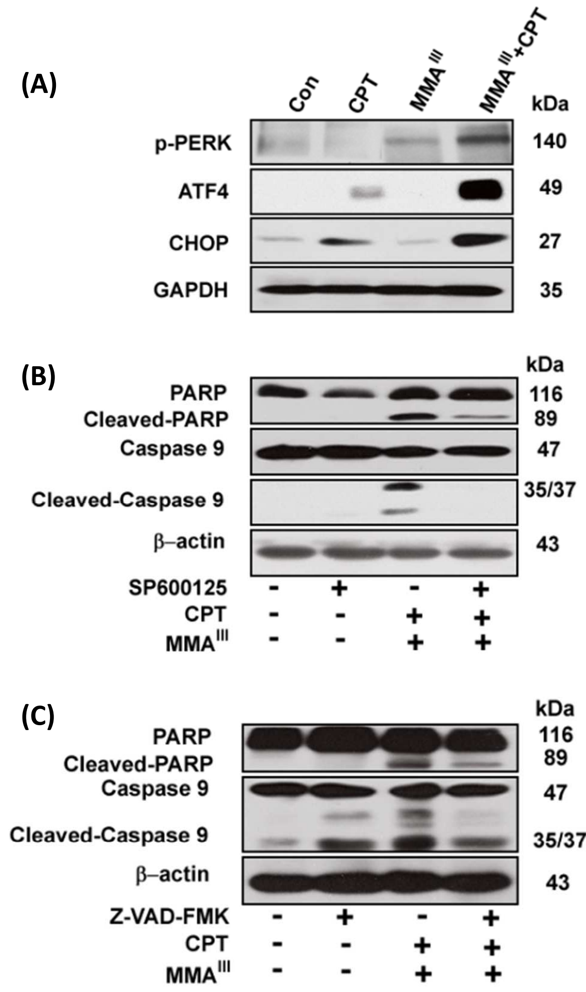

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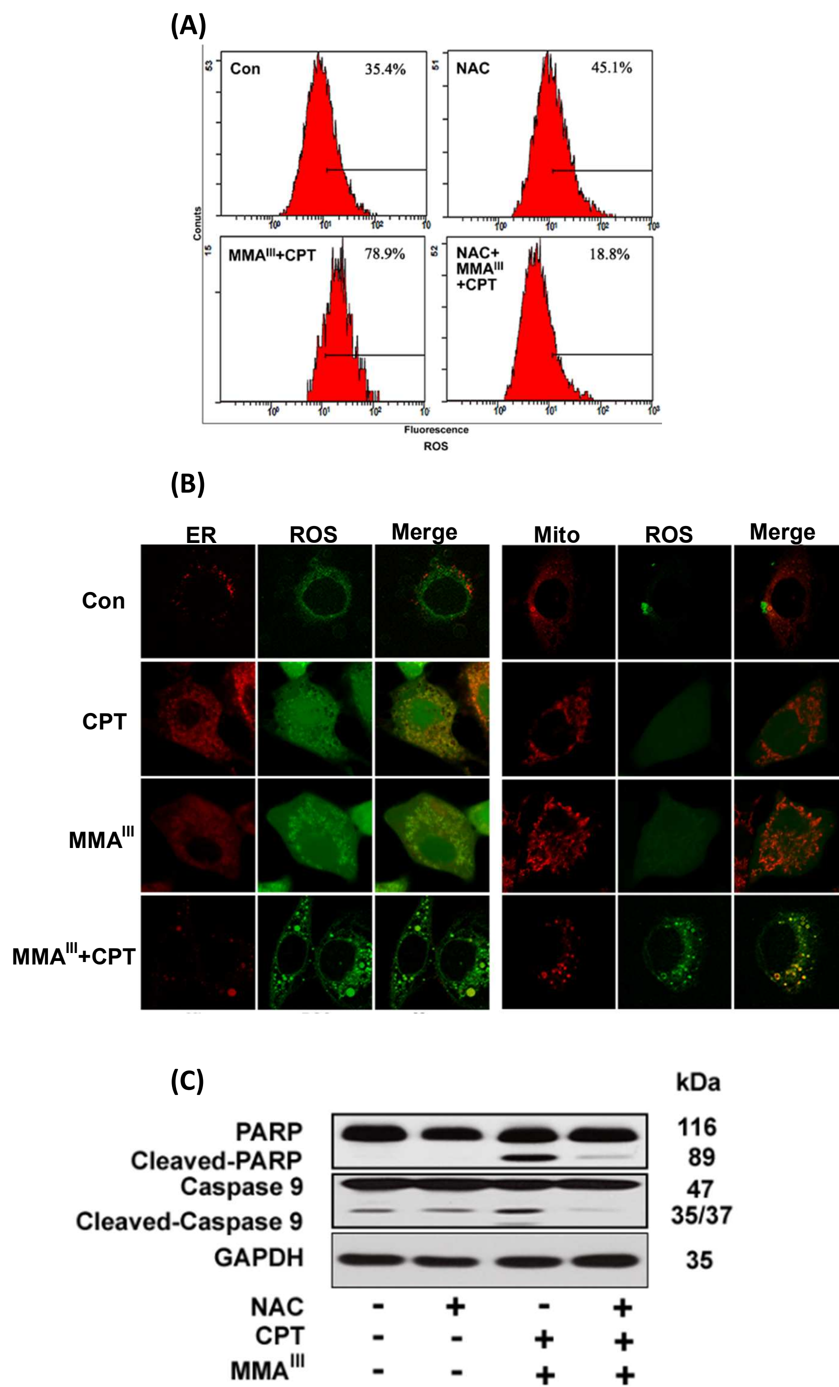
Figure 6



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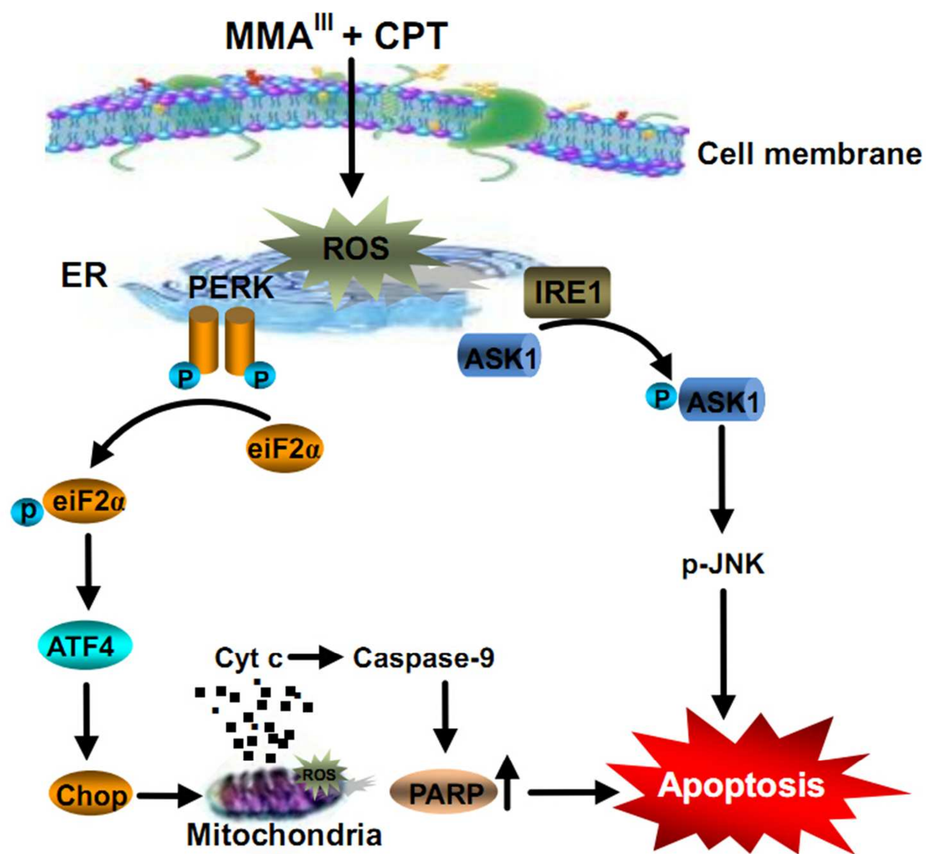

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