International Journal for Pharmaceutical Research Scholars (IJPRS)

V-5, I-2, 2016 ISSN No: 2277 - 7873

RESEARCH ARTICLE

Cytotoxic Study of L-Leucine and Methotrexate Combination in Presence of Super-oxide Dismutase (SOD) on EAC Cells

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Manuscript No: IJPRS/V5/I2/00104, Received On: 27/06/2016, Accepted On: 01/07/2016

ABSTRACT

Our aim was to evaluate the cyto-toxicity of combination of L-Leucine and Methotrexate in presence of SOD (Superoxide dismutase) on EAC cells. Freshly collected EAC cells were sufficiently diluted and was incorporated to evaluate the cyto-toxicity of Methotrexate alone, Methotrexate and L-Leucine in combination and lastly to evaluate *in vitro* cyto-toxicity of Methotrexate, L-Leucine and SOD. The mechanism of action was investigated in terms of production of free radicals. IC_{50} value of Methotrexate was found to be as 95.34 ± 1.28 µg/ml whereas IC_{50} value of Methotrexate and L-Leucine combination 73.15 ± 0.98 µg/ml and IC_{50} value Methotrexate, L-Leucine and SOD was decreased to 47.08 ± 1.18 µg/ml. Due to the fact that Methotrexate is a toxic drug, it will increase the production of O_2^- in cell. Our research hypothesis is based on the fact that decrease of O_2^- by SOD in combination therapy with Methotrexate and L-Leucine may alter its combined cytotoxic effect. So, it can be concluded from the study that presence of SOD in combination therapy with Methotrexate and L-Leucine alters their combined cyto-toxic effects over EAC cells.

KEYWORDS

Methotrexate, L-Leucine, Superoxide dismutase (SOD), Cyto-toxic Study, EAC Cells

INTRODUCTION

Conventional anticancer drug discovery and development have focused on the cytotoxic agents. The drug discovery paradigms selected agents that had significant cytostatic or cytotoxic activity on tumor cell lines and caused regression of tumor. The anticancer agents were discovered mainly by serendipity or inhibiting metabolic pathways crucial to cell division. Their exact mechanisms of action were often a subject of retrospective investigation. For example, Farber et al. reported the use of folate analogues for the treatment of acute lympho-blastic leukemia (ALL) in 1948[1], while its mechanism of action, inhibition of the di-hydro-folate reductase, was reported by Osborn et al. in 1958[2,3]. Similarly, the nitrogen mustard, mustine, was used as a chemotherapeutic agent long before its mechanism of action was understood. Although this strategy has achieved significant success, the recent developments in molecular biology and an understanding of the pharmacology of cancer at a molecular level have challenged researchers to come up with target-based drugs.

Key molecular mechanisms that have been explored for the development of target-based anticancer agents can work by facilitating apoptosis and by inhibiting metastasis. Cytotoxic drug-induced damage to the cells, especially to

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the DNA, triggers apoptosis through two signaling mechanisms – the activation and release of mitochondrial pro-apoptotic proteins known as caspases under the control of Bcl-2 family of proteins or up-regulated expression of pro-apoptotic receptors on cancer cells, whose subsequent interaction with their ligands activates apoptotic signaling pathways. Metastasis is the spread of the tumor from one organ or part of the body to another and is attributed to the translocation of cancer cells. This process of tumor cell translocation requires cellular movement as well as the remodeling of the extra cellular matrix (ECM) that physically entraps cells and defines the shape of a tissue, at both the initial and the metastasized sites of tumor growth and thus the drug candidates targeting proteases and MMP inhibitors have been developed for potential anticancer activity.

Methotrexate is an anticancer drug can be used for the treatment of auto-immune diseases, ectopic pregnancy and also for the induction of medical abortions[4] by acting over the inhibition of folic acid metabolism via Di-hydro-folate-reductase enzyme[5]. Methotrexate is thought to affect cancer and rheumatoid arthritis by two different pathways. For cancer, methotrexate competitively inhibits di-hydro-folate reductase (DHFR), an enzyme that participates in the tetra-hydro-folate synthesis [6, 7]. The affinity of methotrexate for DHFR is about one thousand-fold that of folate. DHFR catalyses conversion of di-hydro-folate to the active tetra-hydro-folate[6]. Folic acid is needed for the de novo synthesis of the nucleoside thymidine, required for DNA synthesis[6]. Also, Folate is essential for purine and pyrimidine base biosynthesis, so synthesis will be inhibited. Methotrexate, therefore, inhibits the synthesis of DNA, RNA, thymidylates, and proteins[7]. For the treatment of rheumatoid arthritis, inhibition of DHFR is not thought to be the main mechanism, but rather multiple mechanisms appear to be involved including: the inhibition of enzymes involved in purine metabolism, leading to accumulation of adenosine; inhibition of T-cell activation and suppression of intercellular adhesion molecule expression by T cells; selective down-regulation of B cells; increasing CD-95 sensitivity of activated T cells; inhibition of methyl-transferase activity, leading to (de)-activation of enzyme activity relevant to immune system function[8,9]. Another mechanism of MTX is the inhibition of the binding of interleukin 1-beta to its cell surface receptor[10]. The most common adverse effects include: hepato-toxicity (liver damage), ulcerative stomatitis, low white blood cell count and thus predisposition to infection, nausea, abdominal pain, fatigue, fever, dizziness, acute pneumonitis, rarely pulmonary fibrosis and kidney failure. Methotrexate is teratogenic (harmful to fetus) and hence not used in pregnancy (pregnancy category X). Central nervous system reactions to Methotrexate have been reported, especially when given via the intra-thecal route (directly into the cerebrospinal fluid), which include myelopathies and leucoencephalopathies. It has a variety of cutaneous side effects, particularly when administered in high doses[11]. Neuro-toxicity may result from the drug crossing the blood-brain barrier and damaging neurons in the cerebral cortex. Cancer patients who receive the drug often nickname these effects "Chemo brain" or "Chemo fog"[(12)]. The aminoglycosides, neomycin and paromomycin, have been found to reduce GI absorption of Methotrexate[13]. Probenecid inhibit methotrexate excretion, which increases the risk of methotrexate toxicity[13]. Likewise retinoids and trimethoprim have been known to interact with methotrexate to produce additive hepato-toxicity and haemato-toxicity, respectively[13]. Other immuno-suppressants like ciclosporin may potentiate methotrexate's haematologic effects, hence potentially leading to toxicity[13]. NSAIDs have also been found to fatally interact with Methotrexate in numerous case reports[13]. Nitrous oxide potentiating the haematological toxicity of Methotrexate has also been documented. Proton-pump inhibitors like omeprazole and anticonvulsant valproate have been found to increase the plasma concentrations of Methotrexate, as have nephrotoxic agents such as cisplatin, the GI drug, colestyramine and dantrolene. Caffeine may antagonise the effects of Methotrexate on rheumatoid arthritis by antagonising the receptors for adenosine.

L-Leucine is an α-amino acid used in the biosynthesis of proteins. It contains an α-amino group and an isobutyl side chain, classifying it as a non-polar (at physiological pH) amino acid. It is essential in humans—meaning the body cannot synthesize it and thus must obtain from the diet. As it is an essential amino acid, animals cannot synthesize leucine. Consequently, they must ingest it, usually as a component of proteins. Plants and microorganisms synthesize leucine from pyruvic acid with a series of enzymes:[14] like Aceto-lactate synthase, Aceto-hydroxy acid isomero-reductase, Di-hydroxy acid de-hydratase, α-Isopropyl-malate synthase, α-Isopropyl-malate isomerase, Leucine amino-transferase. Leucine is an mTOR activator. It is a dietary amino acid with the capacity to directly stimulate muscle protein synthesis. As a dietary supplement, leucine has been found to slow the degradation of muscle tissue by increasing the synthesis of muscle proteins in aged rats [(15)]. However, results of comparative studies are conflicted. Long-term leucine supplementation does not increase muscle mass or strength in healthy elderly men. More studies are needed, preferably ones based on an objective, random sample of society. Factors such as lifestyle choices, age, gender, diet, exercise, etc. must be factored into the analyses to isolate the effects of supplemental leucine as a standalone, or if taken with other branched chain amino acids (BCAAs). Until then, dietary supplemental leucine cannot be associated as the prime reason for muscular growth or optimal maintenance for the entire population. Leucine potently activates the mammalian target of rapamycin kinase that regulates cell growth. Infusion of leucine into the rat brain has been shown to decrease food intake and body weight via activation of the mTOR pathway[15]. Both L-leucine and D-leucine protect mice against seizures[16].

Superoxide dismutase is an enzyme that catalyzes dismutation of the super-oxide (O_2^-) radical into either ordinary molecular oxygen (O_2) or hydrogen peroxide (H_2O_2). Super-oxide is produced as a by-product of oxygen metabolism and, if not regulated, causes many types of cell damage. Hydrogen peroxide is also damaging, but less so and is degraded by other enzymes such as catalase. Thus, SOD is an important antioxidant defense in nearly all living cells exposed to oxygen. Super-oxide is one of the main reactive oxygen species in the cell. As a consequence, SOD serves a key antioxidant role. The physiological importance of SODs is illustrated by the severe pathologies evident in mice genetically engineered to lack these enzymes. Mice lacking SOD2 die several days after birth, amid massive oxidative stress[17]. Mice lacking SOD develop a wide range of pathologies, including hepato-cellular carcinoma,[18] an acceleration of age-related muscle mass loss,[19] an earlier incidence of cataracts and a reduced lifespan. Mice lacking SOD3 do not show any obvious defects and exhibit a normal lifespan, though they are more sensitive to hyperoxic injury[20,21]. Knockout mice of any SOD enzyme are more sensitive to the lethal effects of super-oxide-generating drugs, such as paraquat and diquat. SOD has powerful anti-inflammatory activity. For example, SOD is a highly effective experimental treatment of chronic inflammation in colitis.

Synergistic interactions have been found between Methotrexate and SOD mimetic agents on collagen challenged rats[22]. However due to the fact that Methotrexate is a toxic drug, it will increase the production of O_2^- in cell. Our research hypothesis is based on the fact that presence of SOD in combination therapy with Methotrexate and L-Leucine may alter its combined cytotoxic effect.

MATERIAL AND METHODS

Reagents Used

Commonly used reagents are Methotrexate (Sigma Aldrich, MW: 454.44, Purity: 98%), L L-Leucine (Sigma Aldrich, MW: 131.17, Purity: 98%), SOD (Sigma Aldrich, Purity: 90%), etc. All the other chemicals and reagents used were of high analytical grade.

Collection & Maintenances of EAC Cells

EAC cells were obtained from the recognized laboratories of India. EAC cells were maintained *in vivo* Swiss Albino mice by injecting 0.1 ml of

tumor cell in the intra-peritoneal route. Transplantation of 2×10^6 cells per mouse was done on every 10th day and was drawn out from EAC tumor bearing mouse at the log phase (8th – 9th day of tumor cell inducing) of the tumor cells[23].

Preparation of Phosphate Buffer Saline (PBS)

Phosphate Buffer Saline was used for the dilution purpose because it is non-toxic in nature. 0.312 g Sodium-di-hydrogen phosphate was added to 100 ml of distilled water and 0.356 g Di-Sodium-hydrogen phosphate was added to 100 ml distilled water separately. 81 ml of Di-Sodium-hydrogen phosphate is mixed with the 19 ml of Sodium-di-hydrogen phosphate to produce 100 ml 20 (mM) Phosphate Buffer solution. PH of the solution must be 7.4 and it was checked by using pH meter. Isotonicity testing was done by addition of a single drop blood, followed by centrifugation at 3000 RPM for 10 minutes. Clotting of blood at the bottom of the tube ensures the Isotonicity of the solution. 0.9 g/100 ml of NaCl was added to the phosphate buffer solution for the preparation of the Phosphate Buffer Saline Solution (PBS)[24].

Dilution of Methotrexate, L-Leucine & SOD

Primary concentration of Methotrexate was 500 µg/ 10 µl and it was diluted to 5 µg/ 10 µl by using distilled water. In the similar way L-Leucine and SOD were diluted with distilled water to reach the final concentration 1 µg/ µl in both the cases.

***In Vitro* Cyto-toxicity of Methotrexate**

In vitro cyto-toxicity of Methotrexate was done against Ehrlich Ascites Carcinoma cell. The EAC cells were collected, counted and adjusted to 2×10^6 cells/ml with phosphate buffer saline (0.02M, pH-7.4)/ Krebs Hansleit Buffer. Various concentrations of Methotrexate (10-100 µg/ml) was added and incubated in 37 °C for 1-2 hours. The viability of the cells was determined by Tryphan Blue exclusion method. At the end of 1 hour, the incubated fluid was taken in a WBC pipette and a drop of the diluted cell suspension was placed on the Neubauer's counting chamber and the numbers of cells in the 64 small squares were counted under microscope. The cell viability was determined by adding Tryphan blue (0.4%). Viable cells were found as white in color and the non-viable cells were found as blue in color under microscope. Percentage inhibition was calculated using the standard formulae[25].

***In Vitro* Cyto-toxicity of Combination of Methotrexate and L-Leucine**

*In vit*ro cyto-toxicity of Methotrexate and L-Leucine was done against EAC cells. The EAC cells were collected, counted and adjusted to 2×10^6 cells/ml with phosphate buffer saline (0.02M, pH-7.4). L-Leucine of concentration (10-100 µg/ml) was added with PBS and incubated for 1 hour. After incubation Methotrexate (20 µg/ml) was added to the cells along with PBS and again incubated for 1 hour. The viability of the cells was determined by using Tryphan Blue exclusion method. Incubated fluid was taken in a WBC pipette and a drop of the diluted cell suspension was placed on the Neubauer's counting chamber and the numbers of cells in the 64 small squares were counted under microscope. Percentage inhibition was calculated using the standard formulae[25].

***In Vitro* Cyto-toxicity of Combination of Methotrexate, L-Leucine and SOD**

In vitro cyto-toxicity of Methotrexate, L-Leucine and SOD was done against EAC cells. The EAC cells were collected, counted and adjusted to 2×10^6 cells/ml with phosphate buffer saline (0.02M, pH-7.4). SOD of concentration (10-100 µg/ml) was with sufficient amount of PBS and incubated for 1 hour. After incubation, L-Leucine (50 µg/ml) was added to the cells along with PBS and again incubated for 1 hour. Lastly Methotrexate (20 µg/ml) was added along with PBS and incubation was continued for another 1 hour. The viability of the cells was determined by using Tryphan Blue exclusion method. Incubated fluid was taken in a WBC pipette and a drop of the diluted cell suspension was placed on the Neubauer's counting chamber and the numbers of cells in the 64 small squares were counted under microscope. Percentage inhibition was calculated using the standard formulae[25].

Statistical Analysis

Results are expressed as mean ± SEM & it was calculated by using Graph Pad Prism version 7 Software.

RESULTS

Results of *in Vitro* Cyto-toxicity of Methotrexate

Total number of viable and non-viable cells observed under microscope in each concentration and total number of viable and non-viable cells were calculated (n=8) and Percentage Inhibition value were calculated by using standard formulae. In X-axis Concentration (µg/ml) was plotted and in Y-axis value of Percentage Inhibition was plotted and the graph (Figure: 1) shows gradual increase of Percentage Inhibition in concentration dependent manner. IC_{50} value was found to be as 95.34 ± 1.28 µg/ml.

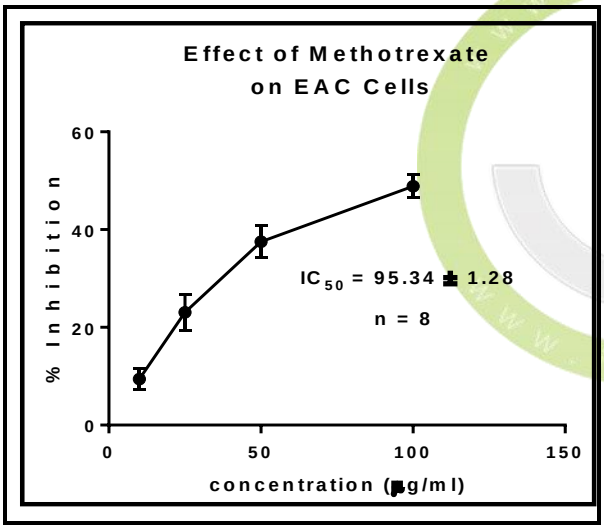

Figure 1: It shows gradual increase of % inhibition value with gradual increase of concentration of Methotrexate. Percentage inhibition was calculated in respect to the number of viable cells and number of non-viable cells found under microscope.

Results of *in Vitro* Cyto-toxicity of combination of Methotrexate and L-Leucine

Total number of viable and non-viable cells and total cell count observed under microscope in each concentration has been counted (n=8) and Percentage Inhibition value were calculated by using standard formulae. In X-axis Concentration (µg/ml) was plotted and in Y-axis value of Percentage Inhibition was plotted. The graph (Figure: 2) shows gradual increase of Percentage Inhibition in concentration dependent manner up to concentration 50 µg/ml and then it obtains a plateau phase. IC_{50} value was found to be as 73.15 ± 0.98 µg/ml.

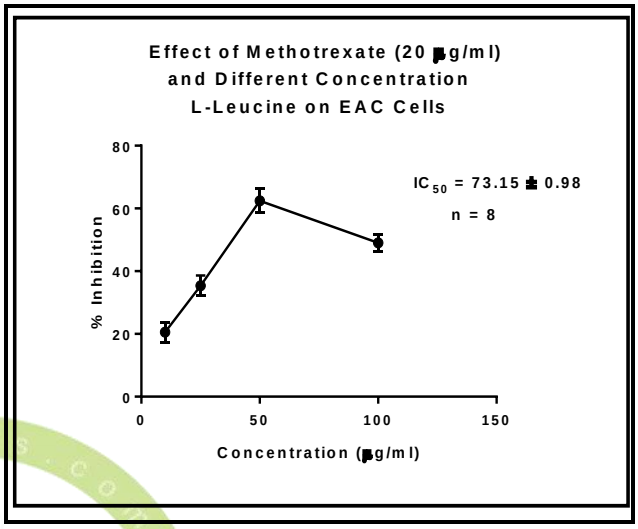

Figure 2: It shows gradual increase of % inhibition value with gradual increase of concentration of Methotrexate & different concentration of L-Leucine up to the concentration 50 µg/ml and after that it reaches plateau phase. Percentage inhibition was calculated in respect to the number of viable cells and number of non-viable cells found under microscope

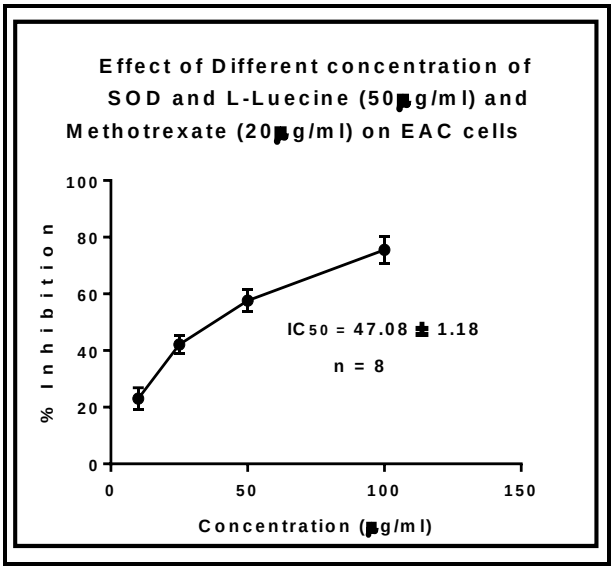

Figure 3: It shows gradual increase of % inhibition value with gradual increase of

concentration of SOD with 20 µg/ml Methotrexate and 50 µg/ml of L-Leucine. Percentage inhibition was calculated in respect to the number of viable cells and number of non-viable cells found under microscope and decrease in IC_{50} confirms the alteration of Cyto-toxicity of Methotrexate and L-Leucine combination in presence of SOD

In Vitro Cyto-toxicity of combination Methotrexate, L-Leucine and SOD

Total number of viable and non-viable cells and total cell count observed under microscope in each concentration has been counted (n=8) and Percentage Inhibition value were calculated by using standard formulae. In X-axis Concentration (µg/ml) was plotted and in Y-axis value of Percentage Inhibition was plotted. The graph (Figure: 3) shows gradual increase of Percentage Inhibition in concentration dependent manner and IC_{50} value was found to be as 47.08 ± 1.18 µg/ml.

DISCUSSION & CONCLUSION

Methotrexate is widely used as a cytotoxic chemotherapeutic agent in the treatment of various malignancies such as acute lymphoblastic leukaemia as well as in the treatment of various inflammatory diseases[26,27]. The efficacy of this agent is often limited by its toxicity which causes severe side-effects and may lead to conditions such as liver cirrhosis, fibrosis of the liver, hypertrophy of the hepatocytes, hepatitis, hepato-cellular necrosis and death[26,28]. It has also been shown that its toxicity has severe side-effects on the haematopoietic system[29] and liver enzymes in general[30]. Methotrexate increases the amount of hydrogen peroxide and other free radicals which are released by stimulated poly-morpho-nuclear neutrophils (PMNs), which may lead to toxicity thus accelerating the rate of cellular damage. It is known that Methotrexate strongly interferes with the metabolism of homocysteine by reducing the levels of 5-methyltetrahydrofolate and as an indirect result, the levels of homocysteine, S-adenosylmethionine (SAM) were also found to decrease[31,32]. It also causes decrease of SAM (SAM acts as an antioxidant) in cerebrospinal fluid of patients on MTX treatment[31,32,33]. Due to its antioxidant effects, a deficiency of SAM caused by Methotrexate may be a reason for increased reactive oxygen species (ROS) and it was shown by Villalobos et al[34]. It is thought that the detrimental effects of Methotrexate are partly due to its direct toxic action by increasing ROS production. It has further been reported that Methotrexate administration induces oxidative stress and significantly reduces antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase in liver, intestinal mucosa and spinal cord tissues of rats[35]. It is however known that cells are protected against oxidative stress by the action of certain enzymes, vitamins, and other substances, collectively known as antioxidants[36].

SOD is the body's primary defense system for tissues, organs against free radicals and this SOD levels decline with age, while free radical production increases. The mechanism of aging is suggested to be related to oxygen free radicals. Free radicals, lipid peroxidation and SOD activity have been reported to be increased in the aged brain[37]. Methotrexate is already a well established anti-cancer drug and the anti-oxidant activity of SOD had also been reported several times. However due to the fact that Methotrexate is a toxic drug, therefore it will increase the production of O_2^- in cell – which leads to the damage of the normal cells. Our research hypothesis is based on the fact that decrease of O_2^- by SOD in combination therapy with Methotrexate and L-Leucine may alter its combined cytotoxic effect. IC_{50} value of Methotrexate was found to be as 95.34 ± 1.28 µg/ml whereas IC_{50} value of Methotrexate and L-Leucine combination 73.15 ± 0.98 µg/ml and IC_{50} value Methotrexate, L-Leucine and SOD was decreased to 47.08 ± 1.18 µg/ml. So, it can be concluded from the study that presence of SOD in combination therapy with Methotrexate and L-Leucine alters their combined cyto-toxic effects over EAC cells.

ACKNOWLEDGEMENTS

I am very much thankful to the NJIL & OMD for their constant support.

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