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Understanding the Efficacy of Anticancer Property of Methylglyoxal along with Indomethacin

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Running Title: Conjugative effect of methylglyoxal and indomethacin against cancer

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Abstract:
Methylglyoxal (MG) is a normal metabolite has long been known for its potency towards anticancer activities byattacking the cancer cellbut without any deleterious effect on healthy normal cells.Thoughthismolecule has been studied since many years against various types of cancer but its degradation by different enzymes existing inside the system is the major concern.The objective of our study is to find out inhibitors of glyoxalaseI, the most potent enzyme for breakdown of MG in order to increase the level of MG within the system. Indomethacin is a non-steroidanti-inflammatory drug (NSAID). The anticancereffect of indomethacin had been known for a long time and has been shown as an inhibitor of glyoxalaseI, and suggested that this anticancer effect might be mediated through augmenting the level of MG. Therefore, in an attempt weexplored the efficacy of anticancer property of MG by using Indomethacin in combination with MGin the present study we observedthat indomethacin has some augmenting effect but at higher level it is quite toxic. Thetoxicity of indomethacin could be moderately ameliorated by methylglyoxal.
Keywords: Methylglyoxal, Indomethacin, Anticancer, NSAID, GlyoxalaseI, Enzyme breakdown

Introduction:

Methylglyoxal (MG), α-ketoaldehydeis a normal metabolite with the formula $CH_3C(O)CHO$. In all mammalian cells, MG is broken down mainly by the glyoxalase system, an enzymatic pathway consisting of two enzymes called glyoxalase 1 (GLO1) and glyoxalase 2 (GLO2) which catalyze the conversion of MG to d-lactate [1] and later the complete metabolic pathway was elucidated [2]. GLO1 amplification and overexpression have been correlated with cancer progression and drug resistance.In 1960s Szent-Gyorgyi proposed a hypothesis on cellular growth regulation and cancer by placing methylglyoxal and glyoxalase I in a central position. [3]But theobstracle with using MG as an anticancer drug is its degradation by various enzymes present in the body.Almost immediately after the anticancer effect of methylglyoxal was reported there began a search or to synthesize inhibitors of glyoxalase I. By *in vitro* experiments several inhibitors of glyoxalase I were actually identified. The synthesis of inhibitors of glyoxalase I was reported as early as 1969 by Vince R and Wadd WB and this research continues till date and a long list of these inhibitors could be presented.[4-6]Not only synthesis, but also a search has been made whether some natural products and existing drugs are inhibitors of glyoxalase I. These drugs are used to treat other diseases and such as plant (turmeric) product curcummin[7], nonsteroidantiinflamatory drug indomethacin [8,9] and a host of other compounds. Moreover many studies have also been made dealing with themolecular mechanism of this inhibition as well as attempt to diminish the level ofglyoxalase I in cells. However it is worthwhile to mention that despite the voluminousliterature that exists on

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inhibitors of glyoxalase I practically very few studies have beenmade on the role and fate of these inhibitors in *vivo* [10].It was hoped that these inhibitors would elevate the level of MG*in vivo* and by this process might augment the anticancer effect of MG. So these inhibitors might act as primary and/or adjuvant chemotherapy against cancer. Mention has also been made that one serious lack in those studies was that very few studies had been done to test the efficacy of these compounds *in vivo* in treating cancer by elevating the level of MG as well as whether these compounds themselves are toxic or not for normal cells.

As mentioned above that as early as 1960s remarkable anticancer effect of MG was observed with in vivo animal experiments. However our lab isthefirst to report of treatment of cancer patients by MG based anticancer formulation as late as 2001 which was published from our laboratory. This and subsequent papers from our laboratory showed very promising results in the treatment of cancer by MG based anticancer formulation both in terms of nontoxicity and efficacy.[11-14] Our laboratory tested the toxicity of MG thoroughly with different animal species through different route of administration at different dose levels and found that MG is potentially safe for human consumption.[11]

MG is a promising anticancer drug improving its efficacy should be animportant agendum in cancer research. MG is a normal metabolite and actedupon by several enzymes *in vivo* to convert it into other products, which have noanticancer effect. So the effective potency of MG is expected to be largelydiminished before reaching the target malignant cells. Of all the MGcatabolyzing enzymes glyoxalase I is the most potent and ubiquitous. So a major field ofresearch is to search and/or synthesis inhibitors of glyoxalase I, which is supposed toaugment the *in vivo* level of MG thereby increasing its anticancer effect.Several years ago the nonsteroidalanti-inflammatory drug indomethacin was found to be an inhibitor of glyoxalase I *in vitro*. The toxicity of indomethacin against a few malignant cell lines had also been shown and it was suggested that the anticancer effect was mediated through inhibition of glyoxalase I [8]. Moreover the molecular mechanism of this inhibition was also studied [9]. A recent study has shown the inhibitory Effects of Indomethacin against human osteosarcoma cell line[15].Simmilarly in an another study a group of researcher showed that indomethacin and juglone induce apoptosis in colon cancer cells by inhibitting inflammatory molecules [16]. MAPK pathway is responsible for a large number of cancers. In a recent article it has been shown that indomethacin can inhibit MAP kinase signal transduction by binding to the phosphotyrosine binding protein Shc and block its interaction[17].Besides its antiinflammatory property, the anticancer effect of indomethacin had been known for quite some time. It has been shown that redox-active copper(II)-phenanthroline-indomethacin complex and redox-inactive zinc(II)-phenanthroline-indomethacin complex can efficiently kill breast cancer stem cells [18].Multidrug resistance is one of the big challenges against cancer chemotherapy. Some studies have revealed the possible solution by making conjugation of indomethacin to form micelle or nanoparticles to overcome those obstacles[19, 20]. However the toxic effect of this molecule had also been known for which it is used in a limited scale. It had also been suggested that this compound might be modified in order to minimize its toxic effect by retaining its anticancer effect [21, 22].

Our idea behind the work is because indomethacin is already used as a drug to treat patients so its use as an adjuvant with methylglyoxal might be relatively easy in comparison to other reported inhibitors of glyoxalase I. So we tested whether the anticancer effect of methylglyoxalcould be augmented *in vivo* by indomethacin and whether the toxic effect of the later could be ameliorated by methylglyoxal.

In this study, we aimed to enhance the anticancer effect of MG by using the inhibitor of glyoxalase I, Indomethacin simultaneously and to check the toxicity, so that this inhibitor might act

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as primary and adjuvant chemotherapy against cancer.

Material Methods:

Chemicals

Methylglyoxalwas obtained from Sigma Chemical Company; St. Louis, MO, USA; ascorbic acid, Na-metabisulphite, gluteraldehyde, Tween 80, acetic acid, sodium sulfate and perchloric acid were from S R L Mumbai, India. Indomethacin was obtained from E.M. PharmacuticalsPvt. Ltd. Mumbai, India, while Diaminobenzene from Merck Specialities Pvt Ltd. Mumbai, India.

Glucose, urea, creatinine, hemoglobin, alkaline phosphatase, serum aspartate transaminase, serum alanine transaminase, creatine kinase and creatine kinase MB assay kits were obtained from Siemens Healthcare Diagnostics Ltd.

Animal treatment

Swiss albino mice aged 4-6 weeks of both sexes were used for the experiments. These animals received normal laboratory diet, and water from common tap present in the laboratory and were housed in the room temperature and humidity of which were maintained at 25-30oC and 55-60% respectively. EAC cells were maintained in the intraperitoneal cavity of mice. The cells were maintained by weekly intraperitoneal inoculation of EAC cells into recipient mice. After 10-12 days, ascites fluid was collected 52 by syringe. The fluid containing EAC cells were immediately diluted with normal saline to avoid coagulation. Erythrocytes, which were found occasionally, were removed by washing with 35mM NaCl. These EAC cells were then used in experiments or to develop and maintain tumor in the intraperitoneal cavity of other mice. Each experimental mouse was treated per day with 0.1ml 0.9% saline containing different amount of MG, and each control mouse received the same amount of sterile sodium chloride (0.9%) by injection through either intravenous or intraperitoneal routes. The animals were also given creatine and ascorbic acid using a 22-gauge ball-tipped feeding needle. Institute's animal ethics committee approved the animal maintenance facility, and the protocol of the experiments.

Tumour growth inhibition study

In this experiment there were 4 groups (6 mice / group). The day on which 1×106 EAC (1 million) cells were inoculated was considered as day 0. The treatment startedfrom day 1 i.e., 24 h after inoculation and continued till day12th.Treatment was formethylglyoxal (intravenous), 20mg/kg body weight/day and for indomethacin (oral)1.5mg/kg body weight/ day. The control group received normal saline intravenously. Allthe groups except control received creatine and ascorbic acid (150mg and 50mg/kg bodyweight/day respectively) orally.

Survival study of multiple doses of indomethacin toxicity andthe protective effects of methylglyoxal

For this experiment all the animals received a particular mode of treatment. Indomethacin was given orally to each mouse whereas methylglyoxal (MG) was injected intravenously through tail vein. Treatment was done for 20 days (4x5 days, 5 days of treatment followed by a gap of 2 days). The dose of MG was 20 mg / kg body weight / day, creatine and ascorbic acid was 150 mg and 50 mg / kg body weight / day respectively through oral route. The animals were kept under observation up to 40 days.

Measurement of body weight

Different amounts (1.5 or 3.0 or 4.5 mg/kg body weight/day) of indomethacin (I) were administered orally either alone or in combination with methylglyoxal (MG), 20 mg / kg body wt/day. Besides, ascorbic acid and creatine 50 and 150 mg /kg body weight/day respectively were fed. Treatment was done for 20 days (4x5 days, 5 days of treatment followed by a gap of 2 days). The control group received only water orally. Each set of experiment was repeated 4 times with six

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animals in each group.

Blood parameters analyses

For different analyses the blood samples were collected from the animals by heart puncture from the different batches of chronic toxicity group. The blood samples were collected 7 days after completion of the treatment. The sera were separated by centrifugation at 2,000 rpm for 5 min. The samples were analyzed for several enzymes and metabolite contents by respective assay kit. Hemoglobin was measured and blood cells counted from a small sample of uncoagulated blood.

Histological studies

Organs from different experimental animals were fixed in 10% buffered formalin and were processed for paraffin sectioning. Sections of about 5 mm thickness were stained with haematoxylin and eosin to evaluate under light microscope.

Results:

Tumor growth inhibition study of methylglyoxal supplemented with indomethacin

We investigated whether mice harboring EAC cells when treated with methylglyoxal supplemented with indomethacin had more pronounced antitumor effect in 97 comparison to treatment by only methylglyoxal. The results are presented in Table 1, which shows that indomethacin has some augmenting effect on the antitumor effect of methylglyoxal. The dose of indomethacin had been fixed from the earlier report of inhibitory concentration of glyoxalase I by this nonsteroidal anti-inflammatory drug and also the results of toxicity study presented below.

Toxicity study of Indomethacin

Toxicity study was carried out with 10 mg/kg body weight/day of indomethacin in mice through oral route for only a single day. All the animals were kept under observation and it was found that indomethacin was very toxic in the above mentioned high dose, as all the animals died within 7 days of the treatment. It was observed that after the treatment with indomethacin in the above mentioned dose, there were toxic effects on physical condition as well as behavioural pattern such as hair texture, food intake and sluggishness, ultimately leading to death.

Toxicity test with multiple doses treatment

For this toxicity study, multiple doses of indomethacin viz. 1.5, 3 and 4.5 mg/kg body weight/day were administered orally to mice up to 20 days (4x 5 days, treatment for 5 days, followed by a gap of 2 days). In the batch with dose level of 1.5mg/kg body weight/day indomethacin, the mice had survived and the survival was followed for 40 days. The animals were healthy, there were no hair loss, no weight loss and no death occurred. There were also no behavioural changes observed during this period. In the dose level of indomethacin 3mg/kg body weight/day batch, death started from 12th day of the treatment. In the dose level of indomethacin 4.5mg/kg body weight/day batch, the animals stopped eating, lost body weight, changed hair texture and became sluggish; death started after 12th day. We investigated the biochemical functions of some vital organs of the animals such as liver, kidney, heart and hemopoietic organs. Treatment continued depending upon the survival of animals. It appears from the biochemical studies that higher doses of indomethacin show some toxic effect on cardiac and hepatic system, which is indicated by the increase in activity of CPK and CPK (MB), SGPT, SGOT and alkaline phosphatase in the serum in comparison to the control animals.(Table 4) From this study, it is assumed that indomethacin is toxic in higher dose level.

Survival study of multiple doses of indomethacin toxicity andthe protective effects of methylglyoxal

As mentioned above, there are reports in the literature that indomethacin is an inhibitor of glyoxalase I. These findings prompted us to study the effect of methylglyoxal on indomethacin

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toxicity and to investigate whether methylglyoxal have any protective effect against indomethacin toxicity. For that, methylglyoxal (20mg/kg body weight/day), ascorbic acid (50mg /kg body weight/day) and creatine (150 mg/ kg body weight/day) were used in conjugation with the above mentioned multiple doses of indomethacin. In the dose level of indomethacin 3mg/kg body weight/day batch, the mice had received methylglyoxal along with ascorbic acid and creatine; death stared after 15 days of treatment. During this treatment, the animals survived for a longer period compared to the batch where only indomethacin was administerd. In the dose level of indomethacin 4.5mg/kg body weight/day batch along with methylglyoxal, ascorbic acid and creatine, the animals showed the symptoms of behavioural and physical changes in the later stages of treatment and have marginal deletrious effect. Death started after 12 days of treatment. The results presented in the table 4 clearly show that methylglyoxal could protect the toxic effect of indomethacin to some extent as indicated by decreased level of CPK, CPK (MB), SGPT, SGOT and alkaline phosphatase in the animals which received indomethacin along with methylglyoxal.The above mentioned results of biochemical studies, measurment of body weight and survival are summarized in Tables 2,3 and 4. As an outcome of this toxicity study, it could be assumed that methylglyoxal has some protective effect on indomethacin toxicity.

Measurement of body weight of mice on treatment with indomethacin alone and in combination with methylglyoxal

Measurement of body weight is a very reliable and authentic index of well-being of animal receiving a drug. Table 3 represents the results of such a study with indomethacin and methylglyoxal. It appears from the table that both the control and the only 1.5 mg indomethacin groups there was not only any loss of body weight but also a marginal increase in body weight indicating that the animals were healthy. Moreover in 1.5 mg indomethacin plus methylglyoxalgroup the results were similar. However in both 3.0 and 4.5 mg indomethacin groups there was progressive loss of body weight and all the animals died and the death of 4.5 mg indomethacin group was earlier. But that methylglyoxal had some protective effect against indomethacin toxicity is observed from the results of 3.0 indomethacin plus methylglyoxal group in which all the animals survived with no loss of body weight.

Effect on several marker enzymes, protiens and metabolites of blood/sera which were subjected to multiple dose toxicity study with indomethacin alone or in combination with methylglyoxal

The results presented above have shown that methylglyoxal could moderately augment the anticancer effect of low dose of indomethacin. Moreover methylglyoxal has some protective effect against the toxicity of indomethacin in the increase in life span and measurement of body weight studies. In the present section we describe the effects of indomethacin alone or in combination with methylglyoxal on some marker enzymes and metabolites of mice. The results are presented in Table 4.

Histological studies of different organs of mice, which were subjected to multiple dose toxicity study with indomethacin alone or in combination with methylglyoxal

Histological studies with several organs of mice were done and the results werepresented in fig. 5 (A&B). For this study repeat dose of indomethacin was treated on micefor different time period. In the dose level of 1.5mg/kg body weight/day of indomethacin and 1.5mg/kg body weight/day of indomethacin plus 20 mg/kg body weight ofmethylglyoxal the treatment was continued up to 15 days because of indefinite survivalof animals. For the dose level of 3.0 and 4.5 mg/kg of body weight alone or plusmethylglyoxal treatment continued up to 7th day because of the lower time period ofsurvival at these dose levels. In all cases creatine and ascorbic acid were feed as usual. Atthe appropriate time the animals were sacrificed and the vital organs

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such as liver, spleen,kidney and stomach were taken for histological study. Although we had observed some deleterious changes in the biochemical studies the histological study shows that there was no major damage in the vital organs of mice during multiple dose treatment with indomethacin.

Discussion

Methylglyoxal is a normal metabolite and metabolized by several enzymes *in vivo* to convert it into other products, which have no anticancer effect. So the effective potency of methylglyoxal is expected to be largely diminished before reaching the target malignant cells. Of all the methylglyoxalcatabolyzing enzymes glyoxalase I is the most potent and ubiquitous. So a major field of research is to search and/or synthesis inhibitors of glyoxalase I, which is supposed to augment the *in vivo* level of methylglyoxal thereby increasing its anticancer effect. Indomethacin had been known for quite some time and different work has shown that it is an inhibitor of glyoxalase I and suggested that the anticancer effect might be mediated through augmenting the level of methylglyoxal, we in this study investigated *in vivo* whether the anticancer effect of methylglyoxal is more pronounced in presence of indomethacin. We observed that indomethacin has some augmenting effect but at higher level it is quite toxic. The toxicity of indomethacin could be moderately ameliorated by methylglyoxal but a significant escalation of the dose of indomethacin was found to beof very limited use for severe toxicity. In the present work we could not however provide any explanation for the decreased toxicity of indomethacin in presence of methylglyoxal, ascorbic acid and creatine; but these compounds have diverse effect in vivo including immunomodulatory role [23,24] for methylglyoxal for which a systematic investigation is needed.

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Conflict of interests

Authors wish to declare that no conflict of interests exist in the publication of this paper.

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EAC Cells in million		
Treatment	Day 0	Day 12th
Control	1	615±12
Indomethacin	1	240±7
MG	1	25±2
MG + Indomethacin	1	11±2

Figure legend

1. In this experiment there were 4 groups (6 mice / group). The day on which 1×106EAC (1 million) cells were inoculated was considered as day 0. The treatment startedfrom day 1 i.e., 24 h after inoculation and continued till day12th.Treatment was formethylglyoxal (intravenous), 20mg/kg body weight/day and for indomethacin (oral)1.5mg/kg body weight/ day. The control group received normal saline intravenously. Allthe groups except control received creatine and ascorbic acid (150mg and 50mg/kg bodyweight/day respectively) orally.

2.For this experiment all the animals received a particular mode of treatment. Indomethacin was given orally to each mouse whereas methylglyoxal (MG) was injected intravenously through tail vein. Treatment was done for 20 days (4x5 days, 5 days of treatment followed by a gap of 2 days). The dose of MG was 20 mg / kg body weight / day, creatine and ascorbic acid was 150 mg and 50 mg / kg body weight / dayrespectively through oral route. The animals were kept under observation up to 40 days.

3. Different amounts (1.5 or 3.0 or 4.5 mg/kg body weight/day) of indomethacin (I)were administered orally either alone or in combination with methylglyoxal (MG), 20 mg/ kg body wt/day. Besides, ascorbic acid and creatine 50 and 150 mg /kg body weight/dayrespectively were fed. Treatment was done for 20 days (4x5 days, 5 days of treatmentfollowed by a gap of 2 days). The control group received only water orally. Each set ofexperiment was repeated 4 times with six animals in each group

4. Different amounts (1.5 or 3.0 or 4.5 mg/kg body weight/day) of indomethacin (I) were administered orally either alone or in combination with methylglyoxal (MG), 20 mg / kg body wt/day. Besides, ascorbic acid and creatine 50 and 150 mg /kg body weight/day respectively were fed. Treatment was done for 20 days (4x5 days, 5 days of treatment followed by a gap of 2 days). The control group received only water orally. Each set of experiment was repeated 4 times with 6 animals in each group.

5. Stains used were hematoxylin and eosin. Magnification Stomach10X, Kidney40X, Liver10X, Spleen 40X.

1. Tumour growth inhibition study of indomethacin supplemented with methylglyoxal

2A. Survival of mice treated with different doses of indomethacin

Treatment group	Number of animals survived up to 20 days	Number of animals survived beyond 30 days
Control (No treatment)	15	15
Indomethacin (1.5mg/kg body wt/day)	15	13

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Indomethacin (3mg/kg body wt/day)	10	03
Indomethacin (4.5mg/kg body wt/day)	08	Nil

2B. Survival of mice with different doses of indomethacin along with methylglyoxal (20 mg / kg body weight / day)

Treatment group	Number of animals survived up to 20 days	Number of animals survived beyond 30 days
Control (no treatment)	15	15
Indomethacin (1.5mg/kg body wt/day) + MG	15	15
Indomethacin (3mg/kg body wt/day)+MG	14	12
Indomethacin (4.5mg/kg body wt /day)+MG	12	6

3. Measurement of body weight and survival of mice treated with indomethacin alone or in combination with methylglyoxal

Body weight (g)								
Mice	Day 1	Day 3	Day 6	Day 9	Day 12	Day 15	Day 18	Day 20
Control	24±0.8	24±1.0	24±1.5	25±0.5	25±0.5	25±1.0	26±1.5	26±1.0
I (1.5 mg)	23±1.0	22±0.5	23±1.0	24±1.0	24±0.5	25±2.0	25±2.0	25±2.0
I (1.5 mg)+ MG	21±1.0	21±0.8	22±0.5	23±0.8	23±0.8	23±1.0	24±0.9	24±1.0
I (3 mg)	24±0.7	22±1.0	23±0.7	22±1.0	20±1.0	died		
I (3 mg)+ MG	20±1.0	20±1.0	21±1.0	21±0.9	22±1.5	22±0.5	23±1.0	23±1.0
I (4.5 mg)	24±0.7	23±1.5	20±2.0	died				
I (4.5 mg)+ MG	20±0.8	20±1.0	21±1.0	21±0.5	22±1.0	died		

4. Biochemical tests of the blood/sera of mice

Test	Control	I(1.5mg)	I(3mg)	I(4.5mg)	I(1.5mg) + MG	I(3mg) + MG	I(4.5mg) + MG
CPK (U/ml)	0.32±0.03	0.35±0.02	1.5±0.02	2.5±0.02	0.31±0.03	0.67±0.04	1.7±0.03
CPK(MB) (U/ml)	0.20±0.01	0.22±0.03	0.64±0.02	1.13±0.04	0.21±0.02	0.51±0.02	0.87±0.04
SGPT (U/ml)	33±2.0	34± 2.5	75± 3	91± 4	33± 2	55± 5	79 ± 2
SGOT (U/ml)	135± 4	122± 6	154± 3	224± 2	124± 3	136± 5	198± 5
Alk.Phos (U/ml)	39± 5	42± 7	129± 7	152± 3	40± 4	111± 5	130± 3
Total Protein (gm/dl)	4.77± 0.3	5.07± 0.2	4.86± 0.2	3.02± 0.1	4.75± 0.3	4.75± 0.4	3.03± 0.3
Albumin (gm/dl)	2.88± 0.4	2.61± 0.2	2.54± 0.2	1.67± 0.3	2.62± 0.3	2.62± 0.2	2.61± 0.4
Total Bilirubin	0.39±0.01	0.48±0.02	0.59±0.02	0.66±0.01	0.46±0.02	0.51±0.01	0.66±0.03

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(mg/dl)							
Urea (mg/dl)	35±3.0	30± 4.0	58± 4.0	74± 3.0	30± 4.0	50± 3.0	66± 2.0
Creatinine (mg/dl)	0.35±0.03	0.34±0.02	0.57±0.03	0.99±0.05	0.27±0.02	0.54±0.04	0.92±0.06

5. Histological study of different organs of mice with
(A) indomethacinalone (B) in combination with methylglyoxal