A BRIEF CRITICAL OVERVIEW OF THE BIOLOGICAL EFFECTS OF METHYLGLYOXAL AND FURTHER EVALUATION OF A METHYLGLYOXAL-BASED ANTICANCER FORMULATION IN TREATING CANCER PATIENTS

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SUMMARY

A historical perspective on methylglyoxal research is briefly presented, mentioning the documented anticancer and antiviral effects of methylglyoxal. The idea and the supporting experimental evidence of Albert Szent-Györgyi *et al.* that methylglyoxal is a natural growth regulator and can act as an anticancer agent are mentioned. Previously a few *in vivo* studies suggested safe administration of methylglyoxal. However, recent literature abounds with the toxic effects of methylglyoxal. The authors present a brief critical overview of studies indicating both toxic and beneficial effects of methylglyoxal and suggest that the beneficial effects of methylglyoxal outweigh its toxic effects. Encouraged by the studies of Szent-Györgyi *et al.*, the present authors undertook systematic investigations to understand the mechanism of the anticancer effect of methylglyoxal. The results of these investigations led to the proposal that the fundamental changes in malignant cells are critical alterations of glyceraldehyde-3-phosphate dehydrogenase and mitochondrial complex I, and methylglyoxal's

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anticancer effect might be mediated by acting on these altered sites. Moreover, a new hypothesis on cancer has been proposed, suggesting that excessive ATP formation in cells may lead to malignancy. Toxicity and pharmacokinetic studies were performed on animals and it was observed that methylglyoxal is potentially safe for humans. A methylglyoxal-based anticancer formulation was developed and a three-phase study of treating a total number of 86 cancer patients was carried out. The results appear to be promising. Most of the cancer patients benefited greatly and a significant number of patients became free of the disease. Contrary to the effect of existing anticancer drugs, this methylglyoxal-based formulation is devoid of any toxic effect and reasonably effective against a wide variety of cancers. The symptomatic improvements of the many patients who died of progressive disease suggest that the formulation could also be used for palliation. The authors urge the scientific community to test the formulation and if found effective then to improve it further.

KEY WORDS

methylglyoxal, ascorbic acid, chemotherapy, cancer treatment, humans, mitochondrial complex I, glyceraldehyde-3-phosphate dehydrogenase, advanced glycation endproducts

INTRODUCTION: HISTORICAL PERSPECTIVE

Interest in the biological role of methylglyoxal began almost a century ago with the identification in 1913 of an enzyme system in animal tissues and in yeast capable of a very rapid conversion of methylglyoxal into D-lactic acid. Moreover, a repeated identification of methylglyoxal among the products of alcoholic fermentation was observed. Thus, in the 1920s, methylglyoxal was widely believed to be one of the key intermediates of glucose breakdown. In the early 1930s, however, different phosphorylated intermediates of the glycolytic pathway were identified, and with the elucidation of the Embden-Meyerhof pathway of glycolysis, the idea that methylglyoxal was a key intermediate of glucose breakdown was abandoned (for review see /1/).

However, despite the lack of any major interest in the biological role of methylglyoxal at that time, from time to time several investigators reported the formation of methylglyoxal in different biological systems. Moreover, there were intermittent reports of several enzymes responsible for the breakdown of methylglyoxal. However, those studies did not culminate in a comprehensive metabolic pathway of methylglyoxal in any organism (for review see /2-4/).

In late 1950s, antiviral /5/ and anticancer /6/ effects of methylglyoxal were first reported. In the 1960s, Szent-Györgyi championed the idea that methylglyoxal might be capable of regulating cellular growth and in turn may act as an anticancer agent /7,8/. Subsequent to this idea there was a gradual revival of interest in the biological role of methylglyoxal. Soon after, the pathways for metabolism of methylglyoxal were elucidated in mammals, yeast and protozoa. The enzymes participating in these pathways were isolated, purified and characterized (for review see /2-4,9/). In the meantime, Szent-Györgyi and his coworkers were able to isolate methylglyoxal from biological samples /10/.

Szent-Györgyi and his collaborators provided strong theoretical arguments and experimental evidence in support of the anticancer effect of methylglyoxal /7,8,10,11/. Együd and Szent-Györgyi showed that when methylglyoxal was injected into mice along with sarcoma 180 cells, no tumour developed and the mice remained completely healthy /11/. At the same time, Apple and Greenberg with remarkable experiments showed that methylglyoxal significantly inhibited tumour growth and in some cases produced indefinite survivors among mice bearing leukemia, lymphosarcoma, adenocarcinoma, sarcoma 180 and other varieties of tumours /12,13/. Other investigators had also observed similar therapeutic activity of methylglyoxal in cancer-bearing animals /14,15/. Moreover, there was no report of adverse toxic effects on the cancer-bearing animals during treatment with methylglyoxal. On the other hand, it was observed that methylglyoxal acts specifically against malignant cells. When exogenous methylglyoxal was added to human leukaemia HL60 cells in culture, inhibition of growth and toxicity was induced; the LD_{50} was 0.24 mM. Methylglyoxal, however, did not induce any toxicity in differentiated cells, neutrophils, under similar culture conditions /16/.

Of all the methylglyoxal catabolising enzymes present in cells, glyoxalase I is the most powerful and ubiquitous /2-4,9/. Several inhibitors of glyoxalase I had been synthesized, and it was found that some of these inhibitors potentiated the anticancer effect of methylglyoxal /17,18/. When *S*-D-lactoylglutathione, a product of glyoxalase I, was added to human leukaemia cells in culture, inhibition of growth and toxicity were induced /19/.

However, in spite of these promising results, till recently neither academic researchers nor clinicians made any attempt to translate this research potential to the treatment of cancer patients. Because methylglyoxal has some growth-inhibitory properties, this apathy probably stems from the belief that methylglyoxal would be toxic. Moreover, there was no systematic study to ascertain whether methylglyoxal acts specifically against malignant cells and spares normal cells. And if that would be the case, then what is the precise mechanism of the anticancer effect of methylglyoxal?

AGE FORMATION BY METHYLGLYOXAL AND ITS BIOLOGICAL EFFECTS

In contrast, recent literature stresses the toxic effects of methylglyoxal. For example, from the mid-1980s, numerous publications have appeared purporting many deleterious effects of methylglyoxal. It was proposed that this toxic effect is mediated mainly through advanced glycation endproducts (AGE) by reaction of the carbonyl groups in methylglyoxal with the amino group present in lysine, arginine and in the terminal amino group of proteins. Moreover, it was also suggested that many diseases, such as diabetes mellitus /20/, cataract formation /21/, hypertension /22/ and uremia /23/, are intimately linked with methylglyoxal-derived AGE formation.

A detailed discussion on this issue is beyond the scope of this paper. In brief, the authors' view in this regard is that the formation of AGE is known to be a non-specific process. Thus, methylglyoxal-derived AGE formation and subsequent pathogenesis might be a very minor event. Moreover, in many cases the results of *in vitro* experiments in non-physiological conditions were extrapolated to *in vivo* situations. Some experiments showed contradictory results and were differently interpreted. We briefly present here some representative

studies on AGE formation by methylglyoxal and some other carbonyl compounds and their biological effects.

Different carbonyl compounds as precursors of AGE

Some examples of AGE compounds are argpyrimidine, pentosidine, carboxymethyl-lysine, glucosepan, DOGDIC, MODIC, GOLD, MOLD, etc. As mentioned above, the formation of AGE is a very non-specific process. For example, several important and indispensable metabolites, such as glucose, glucose-6-phosphate, fructose, ribose, ascorbic acid, etc., are all involved in the formation of AGE /24-29/.

Contribution of methylglyoxal and other carbonyl compounds in AGE formation

Although many studies had implicated methylglyoxal-derived AGE formation with different pathological conditions of the host, in contrast several studies had shown that methylglyoxal plays a very minor role in AGE formation and subsequent toxic effects. In a study to accurately quantify AGE compounds *in vivo*, it was observed that glucosepan, a glucose-derived AGE, was the dominant compound in plasma of normoglycemic individuals (median 17.1 pmol/mg protein), whereas the levels of glucosepan were significantly higher in patients with diabetes mellitus (median 29.2 pmol/mg protein). In contrast, MODIC (a methylglyoxal-derived AGE) levels were found to be in the same range in both the non-diabetic (4.1 pmol/mg protein) and diabetic (3.9 pmol/mg protein) groups. A significant correlation was apparent between values of glycated haemoglobin (HbA_{Ic}) and glucosepan, although MODIC levels were found to be almost independent of HbA_{Ic} concentration /27/. Surprisingly, these investigators could not detect MOLD (another methylglyoxal-derived AGE) in the plasma of either non-diabetic or diabetic individuals, in contrast to the results of other investigators /30,31/. Moreover, in a separate study it was reported that glucosepan is by far the most dominant AGE compound in human skin collagen and glomerular basement membrane in relation to age and diabetes mellitus /29/.

In another study, it was shown that imidazolones, which are the reaction products of the guanidino group of arginine with 3-deoxyglucosone (3-DG), a reactive intermediate of the Maillard reaction, to be epitopes of AGE-modified proteins produced *in vitro*. The 3-DG

level was found to be significantly higher in patients with diabetes mellitus in comparison to healthy individuals. The concentration of 3-DG in erythrocytes was also significantly and positively correlated with HbA_{Ic} /25/. Previously it had also been shown that serum levels of 3-DG were elevated in both diabetic /32/ and uremic /33/ patients. It is to be noted that methylglyoxal has no role in this scheme. Glucose alone can produce 3-DG. Glucose reacts non-enzymatically with protein amino groups to form a Schiff base, which is converted to Amadori rearrangement products, and the latter can then undergo multiple dehydration and rearrangements to produce 3-DG, a highly reactive carbonyl compound. Moreover, monoclonal antibodies raised against the AGE products did not react with the incubation solution of arginine and methylglyoxal or glyoxal /25/, although formation of an imidazolone ring was reported to be present in the incubation solution of an arginine residue and methylglyoxal /34/.

Increased methylglyoxal level in diabetes mellitus and resulting complications

A voluminous literature exists on the relationship of methylglyoxal-derived AGE formation and subsequent diabetic complications /20,35,36/. It was suggested that the increased level of glucose in patients with diabetes mellitus results in increased methylglyoxal formation, which subsequently glycates many proteins, and these altered proteins are primarily responsible for many deleterious effects on the host. However "there is no consensus on the physiological concentration range of methylglyoxal yet" /22/. This comment is corroborated by the results of six studies we refer to below. For example, the plasma concentrations of methylglyoxal in healthy rats were found to be 5 μM /37/ and 14 μM /38/ in two separate studies. Methylglyoxal concentrations of healthy human individuals and patients with diabetes mellitus were also measured. In the four studies that we cite here there is considerable variation in the level of methylglyoxal in the plasma of normal subjects. We present here the values in nM: Odani *et al.* /23/, 650 ± 160; Nemet *et al.* /39/, 520 ± 42; Lapolla *et al.* /40/, 194 ± 11; and Beisswenger *et al.* /41/, 123 ± 37. It is interesting to note that the increase in methylglyoxal level (189 ± 39) in the diabetic patients of one study /41/ is far below the normal level compared to the other two studies /23,39/ and almost equal to that in another study /40/. It is likely that different methods used in the

various studies yielded different results. Only after standardizing the method of methylglyoxal measurement can we can arrive at any definite conclusion.

Cataract formation and methylglyoxal

Metabolism of methylglyoxal and the formation of methylglyoxal-modified proteins has been linked with the development of senile and diabetic cataract /21,42-44/. On the other hand, it has been shown that incubation of bovine and human lens protein digest with 5 mM glucose-6-phosphate or 5 mM glucose led to the formation of fluorescent yellow pigments and cross-links similar to those reported in aging and cataractous human lenses. However, the effects with glucose-6-phosphate were significantly more pronounced. Moreover, the covalent non-disulphide cross-links observed in proteins of senile and cataractous lenses were primarily conjugated with glucose-6-phosphate and far less conjugated with glucose, 17% and 5% of total proteins, respectively /45/. These results suggest that methylglyoxal might have a very minor role, if any, in the development of senile and diabetic cataractous lenses. As methylglyoxal is formed as a glycolytic bypass downstream of glucose-6-phospate, the effects of glucose and glucose-6-phosphate would be expected to be same if methylglyoxal is involved in the process.

Fructose-dependent glycation

In this connection it is worthwhile to mention that some studies have shown that fructose is a superior glycating agent than glucose. For example, *in vitro* experiments have shown that fructose could glycate malate dehydrogenase more efficiently than glucose /46/. In another study, when bovine serum albumin (BSA) was incubated at physiological temperature and pH with either glucose or fructose, formation of carboxymethyl-lysine was observed, and the yield of fructose-modified BSA was up to 17-fold higher than glucose-modified BSA /47/.

Methylglyoxal-derived carboxymethyl-lysine and pathogenesis

Carboxymethyl-lysine (CML), albeit not exclusively, is a methylglyoxal-derived AGE. It has been suggested that CML accumulates in

long-lived tissue proteins with age and it has been implicated in the development of pathology in diabetes mellitus, atherosclerosis /48/ and hypertension /38/. In contrast, it has been suggested that CML is a relatively inert product, both chemically and metabolically, so might have a role in limiting the consequences of protein glycation in the body /49/.

Presence of carboxymethyl-lysine and argpyrimidine in cancer tissues

Using immunological techniques, the presence of both CML and argpyrimidine has been detected in several human cancerous tissues and it has been implicated in the pathogenesis of cancer /50/. However, normal cells upon transformation to malignant cells and with progression of the disease acquire many phenotypic characteristics. Very few of these are specific for cancer. Moreover, as mentioned above, both CML and argpyrimidine are not exclusively methylglyoxal-derived AGE. This proposition is corroborated by the report that when rabbits were fed 1% cholesterol diet and water containing 10% fructose there was marked accumulation of argpyrimidine and carboxymethyl-lysine /51/.

Some potential beneficial effects of methylglyoxal-dependent protein modification

α-Crystallin, a small heat shock protein, prevents protein aggregation under various stress conditions through its chaperone-like properties. Previously, it was demonstrated that methylglyoxal modification of α-crystallin enhances its chaperone function and may thus affect transparency of the lens /52/. Recently it has been demonstrated that besides α-crystallin, methylglyoxal also modifies four client proteins of α-crystallin. It has been suggested that minor modifications of client proteins and α-crystallin by methylglyoxal might prevent protein aggregation and thus help maintain transparency of the aging lens /53/.

Another study observed that methylglyoxal on reaction with an arginine residue rapidly suppresses mitochondrial permeability, thereby preventing the possible deleterious effect of high Ca^{2+} and ganglioside /54/.

In vitro incubation of human erythrocytes with methylglyoxal

During a recent study on the antimalarial activity of methylglyoxal, it was observed that a 4-day treatment of uninfected erythrocytes with 2 mM methylglyoxal caused no morphological abnormalities nor cell lysis, thus showing that methylglyoxal does not produce any random toxic effect /55/. However, in another recent study on human erythrocytes, it was concluded that methylglyoxal impaired energy production and antioxidative defence, enhanced phosphatidylserine exposure of circulating erythrocytes, eventually resulting in anemia and deranged microcirculation /56/.

In vivo effects of methylglyoxal

Despite the assumption of the toxicity of methylglyoxal, very few *in vivo* studies have been carried out on the toxicity of methylglyoxal. In one such *in vivo* study, a single intraperitoneal injection of methylglyoxal (400 mg/kg body wt.) in mice resulted in an increase in aniline hydroxylase activity in liver microsomes after 24 h. Methylglyoxal also decreased glutathione content in the liver, while the activity of serum glutamate pyruvate transaminase was increased. Other changes were also noted. These results suggested to the investigators the onset of liver injuries /57/.

On the other hand, in an *in vivo* study in rats, it was observed that methylglyoxal had a significant protective effect against the ulcerogenic effects on the stomach of several necrotizing agents, such as ethanol, sodium chloride and sodium hydroxide /58/.

Somewhat detailed *in vivo* toxicity studies on animals are available in two relatively recent publications, one by Berlanga *et al.* /59/ and another by Ghosh *et al.* /60/. However, the results of these two studies are apparently contradictory. Berlanga *et al.* noted many degenerative changes on administering 50-75 mg methylglyoxal/kg body weight to rats, five days per week for 7 consecutive weeks. They observed impairment in wound healing; methylglyoxal treatment also arrested growth, increased serum creatinine, induced hypercholesterolaemia and impaired vasodilatation, compared with saline controls. Ghosh *et al.* treated four species of animals, both rodents and non-rodents, with different doses of methylglyoxal via oral, subcutaneous and intravenous routes, and found no toxic effects on the animals (see below).

STUDIES TO UNDERSTAND THE MECHANISM OF THE ANTICANCER EFFECT OF METHYLGLYOXAL

Regarding the mechanism of the anticancer effect of methylglyoxal, Szent-Gyögyi proposed that it is due to its growth inhibitory effect, which in turn is mediated through the inhibition of protein synthesis /61/. In fact, methylglyoxal has been found to be growth inhibitory for a variety of prokaryotic and eukaryotic cells (for other references see /2-4,7,8,61/). However, there was no systematic investigation to understand whether the growth inhibitory effect of methylglyoxal is qualitatively different in malignant cells than its effect on normal cells, although there was a brief preliminary report in this regard /16/.

In 1991, Ray *et al.* observed that methylglyoxal is tumoricidal. When Ehrlich ascites carcinoma (EAC) cells were incubated for 20 min in the presence of 5 mM methylglyoxal, more than 90% of the cells became non-viable. Moreover, when these methylglyoxal-treated EAC cells were inoculated into healthy mice, no tumour developed. It had also been observed that ascorbic acid significantly augmented the tumoricidal effect of methylglyoxal /62/.

The study was extended with a wide variety of post-operative human tissue samples, both normal and malignant, and also with animal tissues and human normal and leukaemic leukocytes. The results of all these studies convincingly demonstrated that methylglyoxal inhibited mitochondrial respiration and glycolysis of malignant cells, but methylglyoxal had no inhibitory effect on mitochondrial respiration of the variety of normal cells tested (with one exception, see below). This inhibition of mitochondrial respiration and glycolysis critically reduced ATP levels in malignant cells rendering them non-viable. Moreover, ascorbic acid significantly augmented the anticancer effect of methylglyoxal. These inhibitions were determined to be via inhibition/inactivation of mitochondrial complex I and glyceraldehyde-3-phosphate dehydrogenase. Experimental evidence has also been obtained which strongly suggests that in malignant cells both these enzymes are critically altered and methylglyoxal acts on these altered sites. As two pathways for ATP generation were found to be strongly inhibited by methylglyoxal, the malignant cells became non-viable /62-68/.

However, in all the normal types of cells tested, methylglyoxal was found to inhibit mitochondrial respiration in only cardiac cells /64/.

Creatine, which is abundantly present in these cells, could completely protect against the inhibitory effect of methylglyoxal. Thus creatine may act against the possible deleterious effect of methylglyoxal on cardiac cells. However, it has been observed that creatine could not protect the mitochondrial respiration of malignant cells, EAC cells, against the inhibitory effect of methylglyoxal /69/.

Based on these results and considering the role of ATP in biological systems, a new hypothesis had been proposed, which suggests that excessive ATP formation in cells may lead to malignancy /70/. This proposed hypothesis conjointly with the results gathered regarding the anticancer effect of methylglyoxal demanded that the efficacy of methylglyoxal in treating cancer patients should be tested urgently. Such testing required and included preclinical studies, such as toxicity and pharmacokinetics, and clinical studies with various types of cancer patients.

TOXICITY AND PHARMACOKINETIC STUDY OF METHYLGLYOXAL

With this intention, Ghosh *et al.* made a thorough investigation on the toxicity and pharmacokinetics of methylglyoxal according to the protocol that is generally suggested for any potential drug intended to be applied to humans /60/.

For this study, four species of animals, mouse, rat, rabbit and dog, were chosen and subjected to methylglyoxal treatment through oral, intravenous and subcutaneous routes. Both acute (treatment for only 1 day) and repeat dose study (treatment for around one month) toxicity tests were carried out. The animals received 0.1-2 g of methylglyoxal/kg body weight/day depending on whether the tests were for acute or repeat dose toxicity study, and also on the routes of administration and the species tested. It was observed that methylglyoxal had no deleterious effect on the physical and behavioural pattern of the treated animals. Studies on several biochemical and haematological parameters of methylglyoxal-treated rats and dogs and histological studies of several organs of methylglyoxal-treated mice were performed. These studies indicated that methylglyoxal had no apparent adverse effect on some vital organs of these animals. Fertility and teratogenicity studies with rats that were subjected to repeat dose toxicity testing showed that these animals produced healthy litters, suggesting no apparent deleterious effect on the reproductive organs of

the treated animals as well as on the offspring. Detailed pharmacokinetic studies were also undertaken in mice after oral administration of methylglyoxal /60/. The study protocols and the results obtained are presented briefly in Table 1.

Ghosh *et al.* also re-investigated the therapeutic potential of methylglyoxal on cancer bearing animals. Previous *in vitro* study had indicated that the anticancer effect of methylglyoxal was augmented in the presence of ascorbic acid /62/. Moreover, endogenously available creatine in cardiac cells could protect these cells from any possible deleterious effect of methylglyoxal /69/. That creatine has some anticancer property had also been previously reported by other investigators /71/.

A PILOT CLINICAL STUDY WITH METHYLGLYOXAL

After the above-mentioned toxicity and pharmacokinetic study, and with the appropriate clearance from the regulatory authorities, Ray *et al.* tested the efficacy of a methylglyoxal-based anticancer formulation in treating a small number of cancer patients /72/. This pilot clinical study was long overdue.

The objectives of the pilot clinical study were to show: 1. whether the formulation taken orally in daily divided doses could lead to tumour shrinkage; 2. whether the formulation could provide pain relief and improve the quality of life in cancer patients; 3. whether the formulation could prolong the life span of cancer patients; and 4. to assess whether the formulation has any toxic effect in cancer patients.

The study design, which included selection criteria for inclusion and exclusion of the patients, composition of the formulation and treatment schedule, and treatment evaluation, was described in detail in our previous publication /73/. The basic ingredients of the formulation are methylglyoxal (25 mg/kg/day) and ascorbic acid (2 g) in four divided doses. Commercially available methylglyoxal was purified by distillation and resin treatment. It was observed that the untreated and treated methylglyoxal had the same *in vivo* effects. Due to the very low yield of methylglyoxal upon purification, methylglyoxal which had not been further purified was used routinely. However, each lot of methylglyoxal was checked for possible toxicity and efficacy by experiments on animals.

The study was divided into three phases. In the first phase (pilot study) of our study, 24 patients were included. The patients were suffering from different types of malignancies: gastrointestinal - five patients; haematological - four; head and neck - three; gynaecological - three; breast - three; liver - two; respiratory organs - one; kidney - one; pancreas - one; gall bladder - one; and other - one patient.

The results of the treatment of the 24 patients indicated that 11 patients were in 'excellent' physical condition, and the condition of five patients was 'good'. Five patients opted out of the study, and three patients died during the course of treatment. We considered the condition of the patients as 'excellent' when the patients were leading an almost normal life and the disease was apparently in remission. 'Good' condition indicated that although the patients had some ailments, these were not life-threatening at that time and their condition was more or less stable /72/.

SECOND PHASE OF TREATMENT OF CANCER PATIENTS

Encouraged by the results of our pilot clinical study, we undertook the second phase of our treatment of cancer patients. For this, a total of 46 patients were enrolled in the study between November 2000 and March 2005. The longest follow up was for 56 months and the average was for 18 months.

It appeared from this study that 35 patients benefited greatly. The formulation was found to be especially effective for cancers of the gastrointestinal tract, ovary, kidney, gall bladder and tongue. Quality of life improved and life span increased for most of the patients, with partial regression and stable disease condition. Even for some patients who had progressive disease, their quality of life was significantly improved.

A detailed description of the 46 patients treated during this phase of our study is given in a previous paper /73/. The description included patient characteristics, history of the disease, and treatment received before the time of inclusion to methylglyoxal treatment, and the outcome on quality of life and disease status of the patients after methylglyoxal treatment. The results of this study indicated that 18 patients were in complete remission, 18 patients had partially regressive disease, and in eight patients the disease was progressive. Two patients were not able to be evaluated.

TABLE 1

Toxicity and pharmacokinetic study with four different species of animals and observed parameters and response to treatment

Animal and different studies	Mode and dose of treatment (g/kg of body weight/day)			Observed parameters / response to treatment
	Oral	Intravenous	Subcutaneous	
Mouse:				
Single for toxicity study (n^a = 6 x 8)	2.0	0.3	1.0	General physical conditions of the animals were observed up to 90 days. No external toxicities (no changes in body weight, hair texture, etc.) and no changes in behavioural pattern were observed
Single for pharmacokinetic study (n = 52 x 6)	0.2	-	-	^{b}lag time (min) ≈30, C_{max} (nmol/ml) - 19.5±3.36, t_{max} (h) ≈ 4, k_1 (h^{-1}) - 0.216, V (l/kg) - 80.97, k (h^{-1}) - 0.192, $t_{1/2}$ (h) - 3.6, CL (l/h.kg) - 15.54
Multiple for toxicity study (n = 6 x 4)	1.0	0.1	0.3	General physical conditions of the animals were observed up to 90 days. No external toxicities (no changes in body weight, hair texture, etc.) and no changes in behavioural pattern were observed.
Multiple for pharmacokinetic study (n = 46 x 3)	0.1	-	-	≈80 h after last dose of methylglyoxal treatment (treated for 30 days): V (l/kg) - 51, k (h^{-1}) - 0.023, $t_{1/2}$ (h) - 30, CL (l/h.kg) - 1.18
Multiple for histological study (n = 20 x 2)	0.5	-	-	Histological examination of liver, kidney, spleen, duodenum and bone marrow showed no adverse effect.
Rat:				
Single for toxicity study (n = 5 x 4)	2.0	0.3	1.0	General physical conditions of the animals were observed up to 90 days. No external toxicities (no changes in body weight, hair texture, etc.) and no changes in behavioural pattern were observed.

Multiple for toxicity study (n = 5 x 4)	1.0	0.1	0.3	General physical conditions of the animals were observed up to 60 days. No external toxicities (no changes in body weight, hair texture, etc.) and no changes in behavioural pattern were observed.
Multiple for biochemical study	1.0	-	-	No significant changes were observed in serum glucose, urea, creatinine and alanine transferase, aspartate transferase, alkaline phosphatase, haemoglobin (whole blood), creatine kinase and creatine kinase (muscle, brain).
Rabbit: Multiple for toxicity study (n = 4 x 2)	0.55	0.1	0.3	General physical conditions of the animals were observed up to 60 days. No external toxicities (no changes in body weight, hair texture etc.) and no changes in behavioural pattern were observed.
Dog and bitch: Multiple (n = 6 x 1)	1.0	0.1	0.3	No significant changes were observed in serum glucose, urea, creatinine and alanine transferase, aspartate transferase, alkaline phosphatase, haemoglobin (whole blood), creatine kinase and creatine kinase (muscle, brain). General physical condition and behaviour remained unchanged.

[a] = number of animals in each group x number of groups.
b = abbreviations of different pharmacokinetic parameters: C_{max} = maximum blood concentration, t_{max} = time to C_{max}, k_a = apparent absorption rate constant, V = apparent volume of distribution, CL = apparent total body clearance, k = elimination rate constant, $t_{1/2}$ = elimination half-life.
These were presented in detail in references /60,74/.

TABLE 2

Present conditions of the 18 patients who were under complete remission in our previous phase II study

Present / Previous Patient Number*	Age/ Sex	Diagnosis	Date of detection	Previous treatment	Commence-ment of the MG treatment	Last medication / last follow-up	Present disease status and QOL (July 2007)
1 / 2	55/ F	**Breast** (adenocarcinoma) recurrence with bone metastasis (diabetic)	October 1999	Surgically treated followed by chemotherapy and radiotherapy	November 2000	August 2003 / till date	Complete remission (81 months) **ND, LN
2 / 3	66/ F	**Gall bladder** (adenocarcinoma) with nodal metastasis (diabetic, pneumobilia, left renal calculus, hypertensive)	December 2000	Surgically treated	February 2001	Continuing with maintenance dose.	Complete remission (78 months) ND, LN
3 / 4	60/ M	**Kidney** (Bilateral renal cell carcinoma)	February 2001	Renal angiography of right kidney and radical nephrectomy of left kidney	May 2001	September 2006	Complete remission up to (59 months) March 2006. April 2006 recurrence in left adrenal gland and lung with pleural effusion. Expired on October 2006

4 / 5	68/ M	**Stomach** (adenocarcinoma) with nodal metastasis	March 2001	Partial gastrectomy and feeding jejujostomy	May 2001	August 2003 / then lost follow-up	Complete remission (28 months) ND, LN till last date of follow-up
5 / 9	85/ F	Urinary bladder (**multiple papillary transition cell carcinoma**)	October 2000	Trans urethral bladder tumour resection (TURBT)	July 2001	March 2007 with maintenance dose / till date	Complete remission (73 months) ND, LN
6 / 13	70/ F	**Breast** (adenocarcinoma) with bone metastasis (diabetic and cardiac patient, degenerative disease)	August 1999	Surgically treated	June 2002	March 2004	Complete remission (22 months) Expired March 2004 due to heart attack ND, DN
7 / 14	62/ M	**Kidney** (adenocarcinoma) (prostatomegaly)	April 2002	Nephrectomy	June 2002	May 2003/ then lost follow-up	Complete remission (12 months) ND, LN till last date of medication
8 / 15	48/ F	**Ovary** (adenocarcinoma)	March 2002	Hysterectomy	June 2002	June 2004/ till date	Complete remission (62 months) ND, LN
9 / 18	52/ F	**Buccal mucosa** (infiltrating squamous cell carcinoma) with nodal metastasis	March 2002	Excised followed by radiotherapy. Local return once with nodal metastasis, again wide excision	October 2002	May 2004/ September 2004 then lost contact	Complete remission (24 months) ND, LN till last date of follow-up

10 / 23	75/ M	**Colon** (adenocarcinoma) with nodal metastasis, ascites, bilateral pleural effusion (prostatomegaly, lumber spondylosis)	March 2003	Exploratory laparotomy followed by hemicolectomy and ileotransverse end to end anastomosis	March 2003	May 2005/ August 2005 then lost to follow-up	Complete remission (30 months) ND, LN till last date of follow-up
11 / 30	50/ F	**Breast** (infiltrating ductal carcinoma) with metastasis in axillary lymph nodes	November 2002	Modified radical mastectomy followed by chemotherapy and radiotherapy	November 2003	August 2004 / then lost follow-up	Complete remission (10 months) ND, LN till last date of medication
12 / 31	72/ F	**Stomach** (adenocarcinoma) Cortical cyst in left kidney	October 2003	Partial gastrectomy with feeding jejunostomy followed by repeated oesophageal dilation	December 2003	Continuing	Complete remission (44 months) ND, LN
13 / 35	56/ F	**Uterus** (adenocarcinoma)	December 2003	Surgically treated followed by radiotherapy	April 2004	September 2005/ till date	Complete remission (40 months) ND, LN
14 / 36	49/ M	**Pancreas** (carcinoid carcinoma) with metastasis to liver and lymph nodes. Primary in colon (diabetic, calculi in gall bladder, cortical cyst in left kidney)	April 2004	Laparotomy, right hemicolectomy	April 2004	Continuing	Complete remission (40 months) ND, LN

15 / 38	68/ F	**Stomach** (adenocarcinoma) with nodal metastasis (diabetic with bi-pedal oedema)	April 2004	Lower radical gastrectomy	May 2004	Continuing with maintenance dose	Complete remission (39 months) ND, LN
16 / 39	54/ F	**Oesophagus** (infiltrating squamous cell carcinoma) with nodal metastasis (calculi in gall bladder)	July 2004	Lower end oesophagectomy and partial gastrectomy	July 2004	Continuing with maintenance dose	Complete remission (37 months) ND, LN
17 / 42	52/ M	**Colon** (adenocarcinoma) with liver and nodal metastasis (hernia)	December 2004	Laparotomy	December 2004	February 2006 / March 2006	Complete remission up to (15 months) February 2006, recurrence in colon in March 2006. Opted out
18 / 43	45/ M	**Brain** (glioblastoma, multiforma)	November 2004	Left temporal craniotomy and decompression of tumour followed by radiotherapy	February 2005	December 2005/ then lost follow-up	Complete remission (11 months) ND, LN till last date of follow-up

* Previous number in Table 2 of reference /73/.
** ND = no tumour; LN = living normally; DN = died due to other disease.

Not all the patients who were inducted into our study kept in contact with us for follow up of their disease status. We only have full records of those patients who maintained regular contact with us. In Table 2 we describe the present condition of the patients who were in complete remission in the second phase of our study. According to the patients' choice, some of them are continuing to take methylglyoxal at a maintenance dose, while for some the treatment has been terminated.

One patient with carcinoma of the kidney (bilateral renal cell carcinoma, patient no. 3 in Table 2) was in complete remission and with excellent quality of life for 58 months. However, after that period the patient developed lung metastasis with plural effusions and died in October 2006.

Another patient with carcinoma of the colon with lymph node and liver metastases was in complete remission up to 15 months (patient no. 17 in Table 2). A recurrence of malignancy occurred at the site of the previous operation and the patient opted out of methylglyoxal treatment.

In the partial remission group of our second phase study, three patients were/are in regular follow up and continued methylglyoxal treatment. Of these three patients, one with adenocarcinoma of the lungs died due to progressive disease. One patient with adeno-carcinoma of the stomach was in remission up to 40 months. Then there was recurrence in the ovary, which was surgically operated. Again she remained stable for another 10 months and after that developed pleural effusions and died due to progression of the disease after 60 months /71/.

THIRD PHASE OF TREATMENT OF CANCER PATIENTS

After the completion of our second phase of study we continued our treatment of cancer patients. This was the third phase of our study, and included 16 patients. The treatment period was from May 2005 to July 2007. The inclusion and exclusion criteria, the dose and mode of treatment were similar to our previous studies /72,73/. In this study the number of patients with each type of malignancy was as follows: gastrointestinal - three; urological - one; gall bladder - two; breast - two; brain - one; gynaecological - one; haematological - two; prostate - one; unknown - two; other - one. The follow up was for 6-26 months. The details of the patients' condition, history of the disease,

and treatment if any at the time of inclusion for methylglyoxal treatment are presented in Table 3.

Table 4 briefly summarizes the outcome of methylglyoxal treatment in the third phase of our study. In this study four patients (patient nos. 1, 3, 5, 6) were in stable condition for a significant period of time without any major complications. However, they died later due to progressive disease and/or other complications. Other patients who are continuing treatment are either in stable condition or are in remission.

DISCUSSION

We started this paper with a brief description of researchers' intense effort at the beginning of the last century to place methylglyoxal on the metabolic map /1/. However, since it was established that methylglyoxal is not a component of the main glycolytic pathway the research on methylglyoxal has been sporadic. Research even at this early phase had shown that methylglyoxal has antiviral /5/ and anticancer /6/ properties. The theoretical arguments and experimental evidence of Szent-Györgyi *et al.* /7,8,10,11,61/ that methylglyoxal can be used as an anticancer agent led to a surge in research on methylglyoxal, and by the end of the 1980s the metabolic pathway of methylglyoxal was firmly established in different organisms /2-4,9/.

Simultaneously, studies on the anticancer effect of methylglyoxal gained momentum. As detailed above, *in vitro* experiments with human and animal tissues and *in vivo* studies in animals should have led to the immediate testing of methylglyoxal to treat cancer patients. However, we had to wait until 2001 to obtain publication of such a study /72/.

The results of the two previous publications on the treatment of cancer patients by a methylglyoxal-based formulation /72,73/ and the present study demonstrated that this formulation is effective against a wide variety of cancers. This observation is similar to the results of the treatment of cancer-bearing animals by methylglyoxal, as reported by Apple and Greenberg and other investigators /11-15/. This efficacy of a single compound/formulation against a wide variety of cancers suggests that different types of malignancies have a common altered site(s), and methylglyoxal acts on these site(s) which are altered specifically in malignant cells.

TABLE 3

Patient characteristics and history of the disease and treatment before the time of inclusion of methylglyoxal (MG) treatment (May 2005 to July 2007) in the third phase of the study (total 16 patients)

P N*	Age /Sex	Type of cancer (Diagnosis)	Other diseases	Time of detection	Previous treatments	Disease status and QOL at start / at presentation	Started MG
1	73 M	**Unknown** Extensive metastatic squamous cell carcinoma in lymph nodes	Prostatomegaly left inguinal hernia, chronic bronchitis for last 20 years	May 2005	None	Extensive metastatic squamous cell carcimona in lymph nodes in left clavicular, paratracheal and intra abdominal region, high prostate specific antigen, prostate volume144ml.	May 2005
2	72 F	**Urinary bladder** Invasive transitional cell carcinoma of the bladder (high grade). Deep muscle invasion	Enlarged liver with cholelithiasis, calculus in gall bladder, diabetic	October 2005	October 2005 Cystoscopy followed by trans urethral bladder tumour resection (TURBT)	Presented with heamaturia, soft tissue density lesion (2 x 1.4 cm) in urinary bladder with adjacent focal wall thickening, deep muscle invasion	November 2005
3	73 F	**Gall bladder** Adenocarcinoma with liver and nodal metastasis.	Multiple calculus in gall bladder, cholelithiasis, choledocholithiasis	December 2005	December 2005 Exploratory laparotomy, inoperable mass in gall bladder	Huge gall bladder mass with multiple hypodense metastatic nodular lesion in liver. Metastatic para aortic and periportal lymphadenopathy	January 2006

4	18 M	**Colon** Poorly differentiated, mucin secreting, infiltrating adenocarcinoma of colon with nodal metastasis and muscle coat infiltration. (stage: Dukes C)		February 2006	February 2006 Anterior resection, partial colectomy and prophylactic colostomy	Marker enzymes for liver function- high	March 2006
5	57 F	**Periampullary carcinoma** (Inoperable) with nodal metastasis		February 2006	March 2007 Exploratory laparatomy followed by gastro-jejunostomy with side to side jejuno-jejunostomy and feeding jejunotomy, common bile duct stenting done.	Poorly differentiated periampullary carcinoma, developed a sinus through feeding jejunostomy line resulting oozing. Retroperitoneal nodes	March 2006
6	51 F	**Acute myeloid leukemia**	Cholelithiasis, splenomegaly	May 2006	None	Weak. Hemoglobin-4.9 g/dl, wbc 3,500 cu mm, blast 50%	June 2006

7	8 M	**Acute lymphoblastic leukemia** (relapsed),		December 2002	December 2002 to January 2005 Chemotherapy. April 2006 Recurrence. Again chemotherapy without benefit	Recurrence April 2006 with metastasis in cerebrospinal fluid. Mediastinal lymphadenopathy with pleural effusion (left), impaired vision, poor general health	June 2006
8	53 F	**Breast** (Bilateral) ductal carcinoma with nodal metastasis	Diabetic, hypertensive, hypothyroid	November 1997	November 1997 Modified radical mastectomy in left breast followed by radiotherapy. June 2006 again radical mastectomy in right breast. Refused to take chemotherapy.	Well differentiated infiltrating ductal carcinoma with nodal metastasis	July 2006
9	71 M	**Stomach** Adenocarcinoma with liver metastasis	Peptic ulcer perforation 20 years back, perinephric abscess 13 years back	October 2005	None	Moderately differentiated adenocarcinoma of stomach with liver metastasis, inoperable, unable to take chemotherapy due to poor health condition. Nodular lesion with friable mucosa in distal part of stomach. Spontaneous bleeding, three-hypodense lesion in liver. Severe pain in abdomen, malena, anorexia, anemia, weakness, unable to move, occult blood in stool, required frequent blood transfusion.	October 2006

10	67 F	**Breast** Ductal carcinoma with bone and nodal metastasis	Chronic cholecystitis	October 2006	November 2006 Mastectomy done	Poorly differentiated ductal carcinoma of breast (left, grade III) with bone and nodal metastasis	November 2006
11	50 F	**Frontoparietal glioma** Right frontoparietal glioma (high grade),	Diabetic, hypertensive, hypothyroid,	November 2006	November 2006 Frontal craniotomy with partial excision of tumor followed by radiotherapy	High frontoparietal SOL, mild subdural haemorrhage and extra axial pneumocephalus. Mild subfalcine herniation towards left side of brain, unable to move upper and lower limbs and deviation of angle of mouth towards right side, poor general health condition, no appetite, sleeplessness, no control in discharging urine.	November 2006
12	80 F	**Endometrium** Adenocarcinoma	Hypertensive, Cholelithiasis	November 2006	None	Moderately differentiated endometrial adenocarcinoma, unable for abdominal hysterectomy due to poor general health condition, bed ridden Haematemesis with fever.	December 2006
13	60 M	**Prostate** (inoperable) Adenocarcinoma with bone metastasis	Renal cyst	June 2006	July 2006 Chemotherapy without any benefit	Ca prostrate with bone metastasis. Adenocarcinoma. Glisson score (5+5) marker enzymes for liver function and prostate specific antigen - high	December 2006

14	67 M	**Gall Bladder** (Inoperable) Adenocarcinoma with liver, colon, duodenum metastasis	Diabetic hypertensive, multiple calculi in lumen of hepatic and bile duct, prostatomegaly grade I	January 2007	December 2006 Exploratory Laparotomy, inoperable- hard lump in liver at the gall bladder fossa and colon, duodenum attached to the lump, closed abdomen without resection or any other manipulation.	Adenocarcinoma in gall bladder involving adjacent liver, colon and duodenum. Pain in abdomen with vomiting and dyspepsia, marker enzymes for liver function - high		January 2007
15	64 M	**Stomach** (Inoperable) Adenocarcinoma with extensive nodal metastasis		December 2006	January 2007 Laparatomy, gastro-jejunostomy and jejuno-jejunostomy done.	Poorly differentiated adenocarcinoma in stomach (inoperable) with retro-peritoneal and omental lymphadenopathy. Poor general health condition. Occult blood in stool.		January 2007
16	62 M	**Carcinoid syndrome** Neuroendocrine tumour with extensive liver metastasis		July 2006	None	Neuroendocrine tumour with extensive liver metastasis. Hepatomegaly with multiple metastatic SOL, thickened gastric wall, pain in whole body, uncontrolled stool discharge		January 2007

*PN = patient number.

TABLE 4

Outcome of quality of life and disease status of 16 patients after methylglyoxal (MG) treatment (May 2005 to July 2007)

P N*	Duration of MG treatment (months)	Date of Last follow up / Medication	Outcome of treatment on quality of life and disease status	Status at last follow-up and comments
1	27	July 2007	Prostate volume reduced to 40 ml April 2007 – Paraaortic and mediastinal lymphadenopathy. Enlarged left supraclavicular and retroperitoneal lymph nodes Prostate specific antigen - reduced Died in July 2007 due to bronchial infection / lung metastasis (?)	Stable disease with average quality of life SD
2	21	Continuing	Heamaturia stopped within two weeks after starting treatment. February 2007- no lesion in urinary bladder, normal wall thickening, gall bladder calculi present	Excellent remission, normal life till date. ND, LN
3	15	March 2007	Initial symptomatic improvement. Stable disease with no lesion in liver. February 2007- patient developed mild ascites and mild right-sided pleural effusion. Enlarged lymph nodes. Expired March 2007 due to progression of disease.	Partial remission, normal life in initial phase. DC
4	17	Continuing	Colostomy bag was removed after two months. Recent reports show no recurrence, no metastasis. Marker enzymes for liver function and tumour marker carcinoembryonic antigen - within normal limit	Excellent remission, normal life till date ND, LN

5	15	May 2007	Symptomatic improvement, no further metastasis, marker enzymes for liver function and blood counts-within normal limit but oozing continued from feeding jejunostomy November 2006 - colostomy bag over the site of sinus. March 2007- marker enzymes for liver function and blood counts-within normal limit but oozing increased due to secondary infection. Expired May 2007 due to septicemia / metastasis (?)	Stable disease, quality of life improved but lately deteriorated for sinus infection SD, DN
6	13	June 2007	Patient received 2 units of blood while starting MG treatment. Then within one month Haemoglobin raised 10.0 g/dl, wbc, plate e count within normal limit. March 2007- blood report within normal, no blast April 2007- raised wbc count and detected blast (2%) in blood. Expired June 2007 due to progressive disease.	Stable disease for 10 months with normal life PD, DC
7	11	April 2007	Good response Initial response was very good with supportive treatment up to February 2007, no pleural effusion, improved general health, gained normal vision with normal blood count. March 2007- wbc count increased to 60,000 /cu.mm. April 2007-Patient opted out.	Stable disease, quality of life normal for 10 months, with some complaint.
8	13	Continuing	No recurrence, no metastasis. Tumour marker Serum CA15-3 (carcinoantigen) within normal limit	Excellent remission, normal life till date ND, LN

9	10	Continuing	Stopped bleeding within a week. No pain, normal appetite, improvement of general health. Blood transfusion was required once during 9 months of treatment. Recent radiological report shows only a small cystic lesion in right lobe of liver	Good response, almost normal life till date RD, LN
10	9	Continuing	No further metastasis	SD
11	9	Continuing	Improvement of general health condition. Can sit, eat, sleep, normal appetite. Recent radiological reports indicate tumour remained stable.	Good response, SD
12	8	Continuing	Symptomatic improvement. Within a week bleeding stopped, remission of fever. Now patient is in ambulating condition, walking capability near normal.	SD
13	8	Continuing	Marker enzymes for liver function and others bio-chemical data are within normal limit. Radiological data (bone scan) indicate improvement Tumour marker prostate specific antigen - reduced	Good response, normal life till date RD, LN
14	7	Continuing	Symptomatic improvement. Occasional supportive treatment required. Pain reduced, no vomiting no dyspepsia. Recent biochemical and radiological data indicate stable disease.	Partial response no mal life till date without any specific complaint. SD, LN
15	7	Continuing	Symptomatic improvement. Biochemical and clinical reports normal, except low hemoglobin percentage, radiologically non-available.	Stable disease with average quality of life. SD
16	7	Continuing	Symptomatic improvement. No pain, no palpable mass, controlled stool discharge. Biochemical reports normal, radiologically not evaluated.	Stable disease, almost in normal life SD

*** PN = patient number.**

Regarding the response of patients to treatment, it was observed in our studies that in addition to improvement, as observed by radiological, biochemical and tumour marker tests, almost all the patients showed an improvement in the quality of life. There was relief from pain and cessation of bleeding. The remarkable feature of this formulation is that it has no adverse side effect. This and the symptomatic improvements of the patients suggest that this formulation can be used also for palliative treatment of cancer patients. The mode of treatment and supplementation of the formulation with some synthetic and/or natural compounds should also be considered. It is expected that intravenous and/or peritumoural injection may significantly improve its efficacy. Supplementation of the formulation with creatine and/or glyoxalase inhibitor might also be tested /17-19,60,71/. Similar to all form of cancer treatment, early diagnosis is advantageous. Because methylglyoxal acts specifically against malignant cells it also has the potential to destroy metastases. However, if vital organs are irreversibly damaged then it is very difficult to bring patients back to normalcy.

However, there are some important limitations of the present and previous studies from our laboratory. There was no statistical analysis of the response to treatment. This is because the patients included in our study suffered from different types of cancer and were at different stages of the disease. Moreover, they received treatment for different time periods. For these reasons a statistical response to treatment could not be performed. However, we have compared the response of our treatment against the usual outcome by conventional treatment.

Another limitation is the inability of defining the time for termination of treatment. Different patients had different types of malignancy and were at different stages of the disease, and their response to treatment was not uniform. Because a malignant cellular mass can regrow from even a single malignant cell, and it is very difficult to identify the presence of a few malignant cells in the body, it is advisable to continue the treatment at a maintenance dose taking into account the fact the methylglyoxal has very little if any toxic effect as compared to that of existing chemotherapeutic agents.

Despite these limitations, the methylglyoxal-based formulation is a reasonably effective non-toxic anticancer formulation, and our primary intention in publishing these papers is to draw the attention of the scientific community to this fact.

A lot has been written recently on the toxic effect of methylglyoxal, which is supposed to be mediated thorough methylglyoxal-derived AGE formation. Without going into a detailed discussion on this issue, we have summarised our opinion and cited some relevant publications. In brief, methylglyoxal-derived AGE formation and subsequent pathogenesis might be a very minor event. Moreover, any drug or even a metabolite has some toxic effect, however little this might be. The effect of a particular compound must be assessed by balancing the benefits and side effects. There are several published papers, including those of Együd and Szent-Györgyi /11/ and Apple and Greenberg /12,13/, that methylglyoxal is well tolerated *in vivo*.

Finally, we appeal to academics and clinicians to test the efficacy of this formulation with a wide number of cancer patients at different centres. There are many anticancer drugs available, which are moderately to highly toxic, and their efficacy is often variable. Only after thorough testing on cancer patients can its efficacy/toxicity be ascertained and further improved. Without testing in cancer patients, mere *in vitro* experiments and *in vivo* studies on animals cannot assess the efficacy of the methylglyoxal-based formulation.

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