

Acute Toxicity and Genotoxic Evaluation of Metlin[®] and Metlos[®] (Organic Agave Fructans)

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Received March 27th, 2013; revised April 27th, 2013; accepted May 7th, 2013

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ABSTRACT

The aim of the study was to contribute to the information on agave soluble fibers since research has been focused on chicory fiber but not in agave products; thus we assess the acute toxicity and genotoxicity of two organic and high purity dietary soluble fibers from agave, Metlin[®] and Metlos[®]. We performed an acute toxicity assay in Hsd:ICR mice and Hsd:Wistar rats and an *in vivo* genotoxic test. Results showed that there are no deaths at any doses or genotoxicity, so it can be concluded that these products are non-toxic, at the administrated doses and none showed a cytotoxic, clastogenic or aneuploidic effect.

Keywords: Agave Fructans; Inulin; Fructooligosacharides

1. Introduction

Chronic diseases prevention has become of great importance for Public Health, research and technology fields, thus one way to do it is through the regular consumption of functional foods.

Food usually refers to any product, supplying to an organism, the energy and chemical substances required for a good health. Nutrients are chemical substances in food that an organism uses and transforms in its own tissues to perform basic functions. Some food contains a certain type of chemical substances capable of causing positive effects to promote and restore health. Functional food implies that the food has some kind of identified value that benefits health, or diminishes the potentiality of disease [1].

In October, 1994, the American Dietetic Association (ADA) adopted a position towards the functional food. The position outlines: "Functional food, including whole food (not modified), and fortified, enriched, improved food have a potentially benefic effect over health when they are consumed as part of a diet in a regular basis, in effective levels. The Association supports a deeper research on this field to define the benefits and risks for health of the individual functional foods and their physio-

logical active components. The professional dieticians will continue working with the food industry, the government, the scientific community, and the media to assure that the public will be able to obtain exact information on this emerging food science field [2]."

When functional food is evaluated in the context of a healthy diet, it must be considered the safe intake levels. The animal research has supplied a close look into this ideal intake; nevertheless this information is difficult to extrapolate to human dietetic requirements. For most of the functional food, the daily intake levels will be established according to clinical assays that have been published in scientific literature.

Most of the functional food will require continued research *in vivo e in vitro*, as well as pharmacokinetic studies, before the specific levels can be determined by clinical assays. Once these clinical assays are completed, the recommendations will be more specific [3].

There exist different types of functional food, but for this matter we will focus on dietary fiber which has some components that can be considered as prebiotics. This type of food must have a synergic relationship with probiotics, and this combination makes possible the benefic effects in the gut [4].

Probiotic refers to a living microorganism that can be

introduced in diet and has a positive effect in health, further more than traditional nutritional effects [5].

Prebiotic refers to the non digestible food ingredients that produce benefic effects on the host, selectively stimulating the growth and/or activity of bacteria in the colon [6]. Dietary fiber refers to different carbohydrates that resist human digestive enzyme hydrolysis, but can be fermented by the colon flora and partially excreted by the feces. This definition would include fibers such as non starch polysaccharides, inulin, fructooligosaccharides (FOS), galactooligosaccharides (GOS) [7,8].

Inulin is a polysaccharide that can be extracted from plants of different families: *Liliaceae*, *Amaryllidaceae*, *Agavaceae*, *Gramineae* *compositae*; although the main source is chicory (*Cichoriumintybus*).

Dietary fiber is an essential part of diet, contributing to the proper functioning of the gut; the recommended fiber intake for adults, according to different countries, are in the range of 25 to 35 grams per day [7,9-13]. Fructans are dietary fibers with prebiotic activity. They are fructose polymers with one or more fructosylfructose linkage [14].

Health Effects of Fructans

The most studied not digestible oligosaccharides (NDO) are FOS and GOS. According to different studies, these compounds have different benefic effects in health:

- Bifidogenic activity. There is a selective stimulation of a limited group of beneficial bacteria in the human colon.
- Short chain fatty acids production and associated effects. NDO fermentation in the colon produces short chain fatty acids, such as acetic, propionic, butyric and also gases (hydrogen and carbon dioxide). These fermentation should give a caloric value of 1.5 kcal/g [15].
- Effect over the number, frequency and weight of fecal stools. It has been observed in animal and human assays that FOS increases the weight of fecal stools [16,17].
- Colon cancer prevention.
- Elimination of diarrhea related to intestinal infections.
- Risk reduction of obesity and type 2 diabetes.
- Effect over Glucemia and Insulinemia.
- Effect over lipids metabolism. A drop in low density lipoproteins (LDL) levels and cholesterol in patients with non insulin dependent diabetes using oligofructose has been described [18,19] and of triglycerides and cholesterol with inuline [20-22].
- Effect over uremia and nitrogen/ammoniac elimination.
- Effect over mineral absorption (Risk reduction of osteoporosis). It is known that in humans, minerals such as Ca, P and Mg are absorbed in the small intestine, but also are effectively absorbed in the colon [23,24].

Several studies on rats conclude that the administration of FOS and GOS stimulate the absorption of Ca, Mg and Fe [25-28].

Agave (*Agavaceae*), called the “tree of wonders”, is a plant species of great use for indigenous groups since prehispanic times. There exist more than 250 agave species. From the plant it is possible to extract food, drinks, animal fodder, construction materials, fabrics and ropes.

Since old times, agave usage has been a constant in the cultural history of Central America; being one of the first crops in the area.

The fermented maguey juice (aguamiel) was called “octli” in nahuatl dialect and “sei” in otomi dialect. Mexican mythology has two versions on its origin: one says that goddess of earth and fertility, Mayahuel, discovered aguamiel and Pachtécatl, her husband, discovered the fermentation process. The second version says that Papantzin discovered the way to extract aguamiel.

Old Mexican cultures appreciated the multiple uses and virtues of aguamiel, even the therapeutic ones. José Zorrilla, a Spanish poet and playwright noted: “Aguamiel is a much appreciated drink, to which Mexicans attribute nutritious and medicinal properties; even they make it drink to weak nursing women because of its capacity to increase milk production [29,30].”

Most of the attributes assigned to agave are due to the presence of fructans on its chemical structure.

Metlin[®] and Metlos[®] are highly purified organic fructans extracted from Blue Agave (*Agave tequilana* Weber).

The main difference between Agave fructans and other types of fructans (mainly inulins) is the linkages β (2-1) y β (2-6) which make them branched molecules. These products are not absorbed in the digestive system and are highly soluble. These characteristics give them nutritional and functional properties providing health benefits. The solubility gives them high competitive advantages, being extensively used as a texturizer and increasing shelf life due to its capacity to retain water.

As pointed before, agave fructans are different from the rest, which makes it a different molecule; also most studies are focused in chicory fructans. Because of this, it is necessary to realize safety and efficacy assessments.

2. Material and Methods

2.1. Metlin[®] and Metlos[®]

Metlin[®] and Metlos[®] are highly purified organic fructans extracted from Blue Agave (*Agave tequilana* Weber); these fructans are reported to be of the branched group with both (β 2→1) and (β 2→6) fructosyl-fructose linkages; their main difference from current commercial fructans is their molecular structure; the structure of several fructan molecules present in Metlin[®] and Metlos[®] which are representative of the highly branched nature of

agave fructans were elucidated by Praznik *et al.* [10]. The main difference between Metlin[®] and Metlos[®] is their degree of polymerization (DP) distribution. Metlin[®] contains mostly fructans over DP 10 and a very low mono and disaccharide content. Metlos[®] contains predominantly fructans under DP 10, with slightly higher monosaccharide content. Since both products consist of ramified molecules, they are extremely soluble in cold water. A major advantage is that these products are the only organic-certified long chain fructans available on the market today.

2.2. Animals

Experiments were performed using certified animals as Specific Pathogen Free “SPF” obtained from Harlan Laboratories-México. Genotoxicity was performed using Hsd:ICR mice 4 - 5 weeks old (Average weight 28 g) and for acute toxicity we used Hsd:ICR mice 4 - 5 weeks old (Average weight 28 g) and Hsd:WI rats 8 - 9 weeks old (Average weight 195 g) [11]. The experiments were conducted after getting prior permission from the Institutional Committee for Care and Use of Laboratory Animals. They were housed in the Animal Experimentation Unit, in the Chemistry School at the National Autonomous University of Mexico, in well-ventilated sterile propylene cages; each cage contained 5 animal of the same sex. There were maintained at a controlled temperature of $22 \pm 2^\circ\text{C}$ and relative humidity $60\% \pm 10\%$ and provided 12 h light/dark cycles. Experiments were started after acclimatizing the animals for one week. They were fed with normal pelleted rodent diet (Teklad 2018S) and water *ad libitum*. The composition of the feed is 18.6% crude protein, 3.5% crude fiber and 6.2% fat.

2.3. Chemicals

Metlin[®] and Metlos[®] were purchased from Nektli S.A. de C.V., México. Mitomycin-C (MMC), Giemsa, Hematoxilin-Eosine solution and Acridine Orange (AO) were obtained from Sigma-Aldrich.

2.4. Preparation of Metlin[®] and Metlos[®]

Metlin[®] and Metlos[®] were dissolved in bidistilled, deionized and filtered water with a 0.22 μm membrane and each one was administered to the animals at different doses in no more than 2 ml/100g of body weight for acute oral toxicity assay.

We used a control group administered with the same quality of water mentioned above. For the genotoxic study the solution was immediately administered intraperitoneally (ip) in a proper volume as showed using Mitomycin-C (powerful clastogen agent) as positive control and PBS as negative control. All treatments are described in **Table 1**.

Table 1. Treatments for acute toxicity and genotoxic assays.

Product	Animal model	Doses mg/kg	Sex	Animals/dose (total animals)	Assay
Metlin [®]	Hsd:ICR mice	17.5	Male	5 (30)	Acute oral toxicity
		55			
		175			
		550			
		1750			
Metlos [®]	Hsd:WI rats	5000	Female	5 (30)	Genoto-xicity assay
		17.5			
		55			
		175			
		550			
Metlin [®]	Hsd:ICR mice	1750	Male	5 (30)	Genoto-xicity assay
		5000			
		143			
Metlos [®]	Hsd:ICR mice	357.5	Male	5 (15)	Genoto-xicity assay
		715			
		143			
Metlos [®]	Hsd:ICR mice	357.5	Male	5 (15)	Genoto-xicity assay
		715			

2.5. Acute Toxicity Studies

Each product (Metlin[®] or Metlos[®]) was administered as a single dose through oral gavage, and the animals were monitored for 14 days. The body weight, mortality, clinical and behavioral symptoms were noted; observations included assessment of fur, eyes, mucosal membranes, respiratory system, activity, with particular attention on possible tremors, convulsions, salivation, and diarrhea.

On Day 14 of the experiment, we proceeded to euthanasia in a CO₂ chamber followed by necropsy for the determination of any possible macroscopically finding and to obtain the next organs: stomach, small intestine (duodenum, jejunum, and ileum), large intestine (cecum, colon, and rectum), and liver. The organs were washed with a 7.4 pH PBS solution and fixed in 10% buffered formaldehyde for the histopathology study. Even the information obtained from acute experiments is unsubstantial; we decided to perform the histopatologic analysis to get more information.

2.6. Genotoxic Studies

2.6.1. Evaluation of Chromosomal Aberrations

After 24 hours of started the treatment, we proceeded to euthanasia in a CO₂ chamber and the femur's bone marrow was extracted from mice. The preparations with the bone marrow cells were stained for 10 minutes with a 5% Giemsa solution (Sigma). The chromosomal aberration

analysis (CA) was performed in a double-blind study in 100 cells in metaphase for each animal. The structural alterations in the chromosomes were scored, both chromosomal and chromatid type following the OECD [31] and EPA [32] procedures.

2.6.2. Micronuclei Assay

Five micro liters of peripheral blood from tail vein of each animal was collected before the sacrifice. Collected blood was placed in the center of an AO-coated glass slide and covered with a 24 mm × 40 mm cover slip. These slides were kept at 4°C for no more than 5 days until analysis. AO supravivally stained erythrocytes and the frequencies of micro nucleated polychromatic erythrocytes (MN-PCE) were examined in a fluorescence microscope [33,34].

2.7. Statistical Analysis

Data from the acute toxicity were analyzed using the statistical SPSS[®] 19 IBM package. Metlin[®] and Metlos[®] were analyzed separately.

A Generalized Linear Model was used in the modality Multinomial logistic regression [35] using, as predictor variables: Treatments, species (Rats, mice) and sex for all kind of lesions in the liver and small intestine (dependent variables). Each lesion has three categories (+slight, ++ moderate, +++sever) so the ordinary logistic link was selected. The Odd Ratios and their Wald Confidence Intervals were obtained for all treatments against the control group.

For the liver, the analyzed lesions were: degeneration and inflammation. For the small intestine the analyzed lesions were: Atrophy and villus fusion and inflammation. The significance level considered in this study was 0.05. It was also obtained the powers of all tests, using the software G*Power 3.1.2, with the following parameters: no centrality parameter $\lambda = 10$, significance obtained with SPSS software, degrees of freedom obtained with the SPSS software.

The weights were analyzed with a polynomial model of repeated measurements, with day as intra-subjects factor and dose and sex as inter-subjects factor, with Greenhouse-Geisser correction for sphericity. In case of differences between groups (doses), a Dunnett measurements comparison test was applied. A significance level of 0.05 was considered for all the analysis, also with statistical SPSS[®] 19 IBM package.

For the genotoxic study, the obtained data was analyzed by comparing the control group against the treated group. A Chi Squarertest [36,37] was used for the analysis of the Chromosome Aberrations frequencies (CA) induced. For Mitotic Index (MI), significant differences were detected with the statistical proportion Z test.

3. Results

3.1. Acute Toxicity

3.1.1. General Status of Treated Groups

All treatments had no important variations observed in body weights during the study and no changes were observed in their physical appearance and behavior.

There were no significant differences ($P > 0.05$, $1 - \beta = 0.69$) attributable to the intake of Metlos[®] or Metlin[®] ($P = 0.10$, $1 - \beta = 0.69$) regarding to weight. All had a significant weight increase ($P = 0.001$); although males were heavier than females in all weight measurements ($P = 0.001$) and these increase is normal due to the growth of the animals.

3.1.2. Histopathology

1) Macroscopic and microscopic description

Gastrointestinal tract of 3 animals per dose per species per product were examined included: stomach, liver, duodenum, jejunum, ileum, cecum, and colon. No significant macroscopic changes were observed. Microscopic changes were subjected to statistical.

2) Liver

No significant differences were found in any the lesions analyzed, attributable to Metlin[®] intake ($P > 0.05$; $1 - \beta > 0.73$) (Table 2). The same results were found to Metlos[®] (Table 3).

Table 2. Liver histopathology results in rats and mice treated with Metlin^{®1} and the control group.

Lesion	Treatment	Specie	Sex
Degrees of freedom	6	1	1
<i>Wald chi-square</i>			
Degeneration	6.50	1.31	3.38
<i>P</i>	0.37	0.25	0.06
$1 - \beta$	0.82	0.90	0.73
Inflammation	3.90	0.22	1.27
<i>P</i>	0.69	0.64	0.26
$1 - \beta$	0.94	0.97	0.90

¹Doses: control, 17.5, 55, 175, 550, 1750, 5000 mg/Kg; ²Test power.

Table 3. Liver histopathology results in rats and mice treated with Metlos^{®1} and the control group.

Lesion	Treatment	Specie	Sex
Degrees of freedom	6	1	1
<i>Wald chi-square</i>			
Degeneration	4.88	0.68	0.002
<i>P</i>	0.56	0.41	0.96
$1 - \beta^2$	0.91	0.94	0.97
Inflammation	1.08	1.82	1.81
<i>P</i>	0.29	0.18	0.29
$1 - \beta$	0.76	0.86	0.91

¹Doses: control, 17.5, 55, 175, 550, 1750, 5000 mg/Kg; ²Test power.

3) Small intestine

In the intestine lesions, no significant differences were attributable to the intake of Metlin[®] ($P > 0.05$, $0.53 \leq 1 - \beta \leq 0.99$) (Table 4) or Metlos[®] ($P > 0.05$, $0.91 \leq 1 - \beta \leq 0.99$) (Table 5).

4) Stomach

No relevant lesion or findings at microscope analysis.

3.2. Chromosome Aberrations

The results from the bone marrow chromosome analysis treated with Metlin[®] and Metlos[®] are shown on Tables 6 and 7.

Data showed that in both treated groups the simple chromosomal aberrations frequency, such as fragments and deletions are within the normal range, when comparing against the negative control group.

In the case of the positive control groups treated with MMC, the chromosomal alteration frequency was substantially higher and statistically significant when compared with the negative control group.

Finally, when the mitotic index was analyzed as a cytotoxicity parameter, it was observed that in none of these treatments the index was modified, with the exception of the group treated with MMC, which decreased significantly.

3.3. Micronuclei Analysis

As an additional parameter for the genotoxic study, an analysis of the micronucleus frequency present in the peripheral blood erythrocytes from mice. The results shown in Tables 8 and 9, indicate that neither Metlin[®] or Metlos[®] increased the micronuclei frequency. The ani-

mals treated with MMC increase significantly when compared with the negative control group.

Table 4. Small intestine histopathology in rats and mice treated with Metlin^{®1} and the control group.

Lesion	Treatment	Specie	Sex
Degrees of freedom	6	1	1
<i>Wald chi-square</i>			
Atrophy and villus fusion	2.21	0.041	0.697
<i>P</i>	0.89	0.84	0.40
$1 - \beta$	0.99	0.99	0.99
Inflammation	5.92	2.69	1.73
<i>P</i>	0.43	0.10	0.18
$1 - \beta$	0.99	0.96	0.98

¹Doses: control, 17.5, 55, 175, 550, 1750, 5000 mg/Kg; ²Test power.

Table 5. Small Intestine histopathology in rats and mice treated with Metlos^{®1} and the control group.

Lesion	Treatment	Specie	Sex
Degrees of freedom	6	1	1
<i>Wald chi-square</i>			
Atrophy and villus fusion	1.09	0.30	0.11
<i>P</i>	0.30	0.58	0.74
$1 - \beta$	0.91	0.99	0.99
Inflammation	2.77	0.01	1.61
<i>P</i>	0.84	0.99	0.20
$1 - \beta$	0.99	0.99	0.96

¹Doses: control, 17.5, 55, 175, 550, 1750, 5000 mg/Kg; ²Test power.

Table 6. Chromosomal alterations frequency in bone marrow cells of mice treated with Metlin[®].

Treatment mg/kg	No. of mice	No. of cells	Del.	Fragm.	Trans.	Gaps	% cells w/+ gaps	% cells w/- gaps	IM
Control	5	500	5	6	-	7	3.6	2.2	4.2
+Control (MMC)	4	400	35	42	4	25	26.5	20.25*	1.1**
143	5	500	7	8	-	9	4.8	3.0	5.4
357.5	5	500	6	7	-	10	4.6	2.6	3.9
715	5	500	4	9	-	8	4.2	2.6	4.0

Del = deletions; Fragg = fragmentations; Trans = translocations; IM = mitotic index; * $P < 0.001$ vs. negative control; ** $P < 0.01$ vs. negative control.

Table 7. Chromosomal alterations frequency in bone marrow cells of mice treated with Metlos[®].

Treatment mg/kg	No. of mice	No. of cells	Del.	Fragm.	Trans.	Gaps	% cells w/+ gaps	% cells w/- gaps	IM
Control	5	500	4	6	-	6	3.2	2.0	3.9
+Control (MMC)	5	500	51	42	6	24	24.6*	19.80*	1.3**
143	5	500	8	8	-	7	4.6	3.2	4.4
357.5	5	500	3	3	1	11	3.6	1.4	3.7
715	5	500	8	6	-	9	4.6	2.8	3.0

Del = deletions; Fragg = fragmentations; Trans = translocations; IM = mitotic index; * $P < 0.001$ vs. negative control; ** $P < 0.01$ vs. negative control.

Table 8. Micronucleus frequency in peripheral blood erythrocytes in mice treated with Metlin[®].

Treatment mg/kg	No. of animals	Micronucleated cells/100cells (X ± E.E.)
Control	5	1.50 ± 0.27
+Control (MMC)	4	6.94 ± 0.90
143	5	1.67 ± 0.37
357.5	5	1.89 ± 0.35
715	5	2.10 ± 0.22

$P < 0.005$ vs. negative control.

Table 9. Micronucleus frequency in peripheral blood erythrocytes in mice treated with Metlos[®].

Treatment mg/kg	No. of animals	Micronucleated cells/100cells (X ± E.E.)
Control	5	1.10 ± 0.031
+ Control (MMC)	5	9.19 ± 0.45
143	5	1.30 ± 0.33
357.5	5	1.10 ± 0.21
715	5	1.67 ± 0.55

$P < 0.001$ vs. negative control.

4. Discussion

This study shows that organic agave inulin and organic agave fructooligosaccharides (Metlin[®] and Metlos[®]), do not cause any toxicity up to the highest dose (5000 mg/Kg). No treatment-related effects were observed in body weight, or microscopic pathology, *in vivo* genotoxic studies have also shown the absence of mutagenic or genotoxic potential. Lack of adverse toxicity seen with these products at this dosage is consistent with a similar lack of significant toxicity exhibited by inulin or fructooligosaccharides from other sources like chicory [38] or a poly- and oligomers of fructose joined by $\beta(2-1)$ fructosyl-fructose bonds called inulin-type fructans [39].

5. Final Comments and Conclusions

There was no death at any doses, concluding that these compounds are not toxic at the administered doses. It is worth mentioning that maximum doses, permitted by internationally accepted protocols (FDA and OECD) were reached: 5 g/kg weight (OECD, 2001-Guideline for testing on acute oral toxicity). The appearance and behavior of mice treated with Metlin[®] and Metlos[®] showed no changes during the study.

The frequency analysis of the chromosome aberrations presented in mice treated with Metlin[®] and Metlos[®] showed that none of each compound are genotoxic, as well as the mitotic index which did not show a cytotoxic effect.

The frequency of micronuclei presence in polychro-

matic erythrocytes confirmed the fact that none of the compounds tested are clastogens (chromosome-breakers) or induced aneuploidies.

There were no significant histopathology changes in any of the organs studied.

In conclusion, we can say that under the model and experimental conditions used, both Metlin[®] and Metlos[®] are non toxic, cytotoxic or genotoxic. The further validation using clinical trial is still needed.

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