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## Evidence that isopropylthioxanthone (ITX) is devoid of anxiolytic and sedative effect

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**Evidence that isopropylthioxanthone (ITX) is devoid of anxiolytic and sedative effect**

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3 **Evidence that isopropylthioxanthone (ITX) is devoid of anxiolytic**  
4 **and sedative effect**  
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For Peer Review Only

### Abstract

Isopropylthioxanthone (ITX) is a well known photoinitiator in UV-cured inks frequently used in milk packaging materials, yoghurt, ready-to-feed infant formula and other drinks. Traces of ITX has been found in milk and, as a consequence, there was considerable interest in studying the biological activity of this molecule and its potential hazard for the human health. Although the ITX genotoxic effects have been excluded by the European Food Safety Authority (EFSA), the Environmental Protection Agency (EPA) in USA is still examining its possible toxic potential depending on a dose-effect ratio. Little is known about the ITX activity on the function of the Central Nervous System (CNS) and the cerebral neurotransmitters. Using behavioral, biochemical and electrophysiological tests, we have found that: (a) ITX did not exert *in vivo* anxiolytic or sedative effect, when administered orally to rats; (b) Did not affect the binding characteristics of central and peripheral benzodiazepine receptors studied *in vitro*; (c) Did not influence the ability of GABA to increase the chloride channel permeability studied by patch clamp technique in single neuron of cultured cerebellar granule cells.

**Keywords:** Isopropylthioxanthone (ITX), benzodiazepine receptors, anxiety, sedation, electrophysiology

## Introduction

In November 2005, 30 million litres of baby milk were recalled from the market in Italy, France, Spain and Portugal after an announcement from the Italian authorities to the European Commission via a Rapid Alert System for Food and Feed (RASFF) notification. Some batches of liquid baby milk contained up to  $250 \mu\text{g L}^{-1}$  (ppb) of ITX (Fig.1), a photoinitiator used in printing inks for offset printing on the outside of beverage cartons [RASFF Annual Report, 2005]. Its presence in milk was accidentally due to the manufacturing process in which the multilayer cardboard was rolled on reels and the substance was then transferred from the outer printed surface to the inner plastic surface prior to the construction of the individual cartons [RASFF Annual Report, 2005].

The European Food Safety Authority (EFSA), was requested by the European Commission to give an Opinion, and noted that levels of ITX in dairy products varied from 27 to  $445 \mu\text{g/L}$  and from 5 to  $249 \mu\text{g/L}$  in fruit juices, fruit nectars and drinks [EFSA, 2005]. In addition a German research institute also published positive findings of ITX in TetraPack fruit juices of  $405 \mu\text{g/kg}$  [Rothenbacher et al., 2007]. At the end of 2005, EFSA stated that the presence of ITX in food does not raise health concerns, so the Standing Committee decided to close the survey and to take no measures.

Little is known about ITX toxicity and its general biological activity, only a few studies refer that ITX seemed to have limited genotoxicity *in vitro* [Kirkland et al., 2005], but literature is lacking in certain proofs. ITX was tested *in vivo* in the mouse bone marrow micronucleus test [Notox, 2004a] and subsequently tested for its ability to induce unscheduled DNA synthesis in rat liver using an *in vivo/in vitro* procedure [Notox, 2004b]. In the first experiment, treatments were well tolerated with no signs of systemic toxicity, no increase in the incidence

1  
2  
3 of micronucleated PCEs and no decrease in the ratio of polychromatic to normochromatic  
4 erythrocytes in treated animals compared to vehicle controls [Notox, 2004a]. Then in the  
5  
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7 second experiment, treatments did not produce clinical signs at any time, no increase in mean  
8  
9  
10 Net Grain Count (NNG) value and in the incidence of cells in repair (with NNG >5) was  
11  
12  
13 observed in rats treated with ITX compared to the vehicle group [Notox, 2004b].

14  
15 Momo et al. (2007) demonstrated that ITX changes the lipid structure in multilamellar  
16  
17 liposomes of saturated phospholipids of different length with and without cholesterol. As a  
18  
19 matter of fact in the gel state, ITX acts as a disordering agent, increasing the mobility of the  
20  
21 bilayer; while in the fluid state, it tends to rigidify the membrane, in a cholesterol like way  
22  
23  
24 [Momo et al., 2007].  
25  
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29 During a meeting of CODACONS (private association for the defence of the consumer and  
30  
31 the environment) in Modena (Italy) at the end of 2006, it was stated that ITX probably act as a  
32  
33 benzodiazepine-like (BDZ) compound [CODACONS, 2006]. In addition, at the end of  
34  
35 February 2009, a judge of Giarre (Catania, Italy) awarded against Nestlè and Tetrapack,  
36  
37 compensation to two families for the psychological damage caused by the concern that their  
38  
39 daughters might be contaminated by ITX present in the milk they drank [CORRIERE DELLA  
40  
41 SERA, 2009].  
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48 The aim of the present study was to establish whether or not ITX exerts an anxiolytic-sedative  
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50 effect and if it acts as a benzodiazepine-like compound.  
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## Materials and methods

### 1. Behavioral studies

#### 1.1. Animals

Adult male Sprague-Dawley rats (Harlan Italy, Udine, Italy) weighing  $250 \pm 1.9$  g were used throughout the experiments. They were housed (2 rats each cage) in a controlled environment (air-conditioned room at  $22 \pm 1^\circ\text{C}$  and 60% humidity) maintained under a 12:12 h light/dark cycle. Rats had free access to water and food, a standard pellet containing water 10.68%, crude proteins 21.12%, crude fats 4.22%, fibers 4.11%, ashes 6.61% and other components 53.26% (Mucedola s.r.l., Settimo Milanese, Milano, Italy).

All procedures were performed in accordance with the guidelines of the National (D.L. no. 116/1992) and European legislation (EEC no. 86/609) and of the National Institute of Health on the use and care of laboratory animals. Behavioral experiments were conducted from 10:00 a.m. to 2:00 p.m.

#### 1.2. Treatments

ITX (Isopropyl-9H-thioxanthen-9-one, mixture of 2- and 4-isomers; CAS 75081-21-9, purity 97%; Sigma-Aldrich Italy, Milano, Italy) was solubilized in Tween 80 (1%) and water and then administered at the doses of 1, 10 and 25 mg/kg in a volume of 5 ml/kg body weight (b.w.), by oral gavage, 60 min before tests. The control groups received the vehicle treatment (Tween 80 1% and water).

Pentobarbital sodium (Carlo Sessa S.p.A., Milano, Italy) was injected intraperitoneally (i.p.) at the dose of 35 mg/kg in a volume of 2 ml/kg b.w., 60 min after the treatment with the tested compounds.

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2  
3 Diazepam (Valium®; Roche S.p.A., Monza, Milano, Italy), used as reference drug in the  
4  
5 elevated plus maze test, was i.p. injected at the dose of 1 mg/kg, 20 min before the test; in the  
6  
7 open field test it was i.p. injected at the dose of 2 mg/kg, 20 min before the test.  
8  
9

### 10 11 12 1.3. Elevated plus maze test

13  
14 The elevated plus maze was a wooden apparatus consisting of two opposite open arms (50 cm  
15  
16 long, 10 cm wide) and two opposite closed arms of the same size, enclosed by 40 cm high  
17  
18 walls. The apparatus was elevated 50 cm above the floor and illuminated from the top. A  
19  
20 video camera was suspended above the maze to record trials for analysis. Following the  
21  
22 procedure described by Pellow et al. [Pellow et al., 1985], rats were individually placed in the  
23  
24 central square facing an open arm and observed for 5 min.  
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32 The number of entries and the time spent in the open and closed arms were recorded. The  
33  
34 maze was cleaned following each trial to remove any residue or odour of the animals. For the  
35  
36 purpose of analysis, open (or closed) arms activity was quantified as the percentage of  
37  
38 number of entries into open (or closed) arms relative to the total number of entries into any  
39  
40 arm and the percentage of the time spent in the open (or closed) arms relative to the total time  
41  
42 spent in the maze.  
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### 48 1.4. Open field test

49  
50 For the test a black painted arena 100 cmx100 cm square floor and 50 cm high walls was  
51  
52 used. The test started by gently placing each animal in the centre of arena. Rats were  
53  
54 continuously filmed for 10 min with a camera connected to a computerized system  
55  
56 (Biomedica Mangoni s.n.c., Pisa, Italy), which divided the field into 25 squares of equal size.  
57  
58 Sixteen squares delimited the peripheral area and nine squares the central area. The length of  
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1  
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3 the rat pathway in the total open field as well as in the peripheral and the central areas, and the  
4  
5 number of rearings (when the rat reared upon its hind feet) were recorded. At the end of each  
6  
7 trial, the open field was wiped clean to remove traces of the previous assay.  
8  
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### 10 11 12 13 1.5. Pentobarbital-induced sleeping time

14  
15 A single dose of pentobarbital sodium (35 mg/kg) was i.p. injected in rats 60 min after the  
16  
17 oral administration of the vehicle or ITX. Sleeping time was determined as the interval  
18  
19 between the loss and the recovery of the righting reflex.  
20  
21

## 22 23 24 25 2. Radioligand binding assay studies

### 26 27 28 29 2.1. Sample preparation

30  
31 ITX (Sigma-Aldrich Italy) was dissolved in methanol (Carlo Erba Reagenti, Milano, Italy) to  
32  
33 obtain the  $10^{-2}$  M ITX-standard solution. The standard  $10^{-2}$  M ITX-standard solution was  
34  
35 diluted with sodium phosphate monobasic monohydrate buffer (Sigma-Aldrich Italy) 50 mM  
36  
37 pH 7.40 or Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (Sigma-Aldrich  
38  
39 Italy) buffer 50 mM pH 7.70 to obtain the experimental ITX-solutions from  $10^{-4}$  M to  $10^{-9}$  M.  
40  
41  
42  
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### 45 46 2.2. Central benzodiazepine receptor competition binding assay

47  
48 The activity of ITX-solutions from  $10^{-4}$  M to  $10^{-9}$  M on the central benzodiazepine (BDZ)  
49  
50 recognition binding site, was evaluated by a radioligand binding assay using [ $^3$ H]RO 15-1788  
51  
52 (8-fluoro-3-carboethoxy-5,6-dihydro-5-methyl-6-oxo-4-himidazo[1,5-a]1,4 benzodiazepine)  
53  
54 (specific activity 3.09 TBq/mmol; NEN<sup>TM</sup> Life Science Products, PerkinElmer Italy, Monza,  
55  
56 Milano, Italy) as specific ligand. Aliquots of ITX-solutions were tested for their ability to  
57  
58 inhibit 1 nM [ $^3$ H]RO 15-1788 specific binding to rat cerebellar membranes prepared as  
59  
60

1  
2  
3 previously described from Olasmaa [Olasmaa et al., 1990]. Rat cerebellar membranes (100 µl,  
4  
5 500-1000 µg of protein/ml), re-suspended in sodium phosphate monobasic monohydrate  
6  
7 buffer, were incubated with 1 nM [<sup>3</sup>H]RO 15-1788 (25 µl) and aliquots (100 µl) of ITX-  
8  
9 solutions or 1 µM unlabeled diazepam (Roche S.p.A.) to assess non-specific binding. The  
10  
11 final volume (250 µl) was obtained by adding sodium phosphate monobasic monohydrate  
12  
13 buffer.  
14  
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16  
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18 The mixture was incubated at 0-4 °C for 1 h. The reaction was terminated by vacuum  
19  
20 filtration through glass fibre filters (Whatman GF/C; Whatman International Ltd, United  
21  
22 Kingdom) followed by three washings with 3 ml of ice-cold sodium phosphate monobasic  
23  
24 monohydrate buffer. The filters were transferred to vials with liquid scintillation cocktail (3  
25  
26 ml) and radioactivity was measured in an LS 1701 Rack Beta liquid scintillation counter  
27  
28 (Beckman Coulter S.p.A., Milano, Italy). Protein concentration was determined by the  
29  
30 method of Bradford [Bradford, 1976] with Bovine Serum Albumin (BSA) used as a standard.  
31  
32 The activity of the ITX solution was based on the displacement of [<sup>3</sup>H]RO 15-1788 from  
33  
34 cerebellar membranes. This activity was quantified by comparison with a competition curve  
35  
36 generated with diazepam at the same concentration of ITX-solutions.  
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### 46 2.3. Peripheral benzodiazepine receptor competition binding assay

47

48 The activity of ITX-solutions on peripheral benzodiazepine (BDZ) receptor was evaluated by  
49  
50 a radioligand binding assay using [<sup>3</sup>H]PK 11195 (specific activity 3.18 TBq/mmol; NEN<sup>TM</sup>  
51  
52 Life Science Products, PerkinElmer Italy) as specific ligand. Aliquots of ITX-solutions were  
53  
54 tested for their ability to inhibit 1 nM [<sup>3</sup>H]PK 11195 specific binding to rat adrenal glands  
55  
56 membranes prepared as previously described from Anholt [Anholt et al., 1986]. Adrenal  
57  
58 glands membranes (160 µl, 100 µg of protein/ml) resuspended in Tris-HCl buffer were  
59  
60

1  
2  
3 incubated with 1 nM [<sup>3</sup>H]PK 11195 (20 µl) and aliquots (20 µl) of the ITX-solutions or 1 µM  
4  
5 unlabeled PK 11195 (Sigma-Aldrich Italy) to assess non-specific binding. The mixture was  
6  
7 incubated at 0-4 °C for 1 h. The reaction was terminated by vacuum filtration through glass-  
8  
9 fibre filters (Whatman GF/C, Whatman International Ltd) presoaked in 0.5%  
10  
11 polyethyleneimine (Sigma Aldrich Italy) followed by three washings with 3 ml of ice cold  
12  
13 Tris-HCl buffer. The filters were transferred to vials with liquid scintillation cocktail (3 ml)  
14  
15 and radioactivity was measured in an LS 1701 RackBeta liquid scintillation counter  
16  
17 (Beckman Coulter S.p.A.). Protein concentration was determined by the method of Bradford  
18  
19 [Bradford, 1976] with BSA used as a standard. The activity of the ITX solution was based on  
20  
21 the displacement of [<sup>3</sup>H]-PK11195 from adrenal glands membranes. This activity was  
22  
23 quantified by comparison with a competition curve generated with unlabeled PK11195 at the  
24  
25 same concentration of ITX-solutions.  
26  
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#### 34 2.4. Central benzodiazepine receptor saturation binding assay

35  
36 In saturation binding assays, increasing concentrations of [<sup>3</sup>H]RO 15-1788 (25 µl, 0.37–12  
37  
38 nM) were incubated with rat cerebellar membranes (100 µl, 500-1000 µg of protein/ml)  
39  
40 prepared as previously described from Olasmaa [Olasmaa et al., 1990], with and without ITX  
41  
42 (25 µl at the dose of 10<sup>-4</sup> M). The final volume (250 µl) was obtained by adding sodium  
43  
44 phosphate monobasic monohydrate buffer. Non-specific binding was measured in the  
45  
46 presence of diazepam 1 µM.  
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53 The mixture was incubated at 0-4 °C for 1 h. The reaction was terminated by vacuum  
54  
55 filtration through glass fiber filters (Whatman GF/C, Whatman International Ltd) followed by  
56  
57 three washings with 3 ml of ice-cold sodium phosphate monobasic monohydrate buffer. The  
58  
59 filters were transferred to vials with liquid scintillation cocktail (3 ml) and radioactivity was  
60

1  
2  
3 measured in an LS 1701 Rack Beta liquid scintillation counter (Beckman Coulter S.p.A.).  
4  
5 Protein concentration was determined by the method of Bradford [Bradford, 1976] with BSA  
6  
7 used as a standard. The activity of the ITX solution was based on its ability to act as an  
8  
9 allosteric modulator to enhance or inhibit the effects of diazepam.  
10  
11

### 12 13 14 15 *3 Electrophysiological studies*

#### 16 17 18 3.1 Primary cultures of cerebellar granule cells

19  
20 Primary culture of cerebellar granule neurons were prepared from 7 days old Sprague-  
21  
22 Dawley rats as previously described (Gallo et al. 1987; Luo et al 2002) following the  
23  
24 European Community Guidelines for the use of experimental animals. Briefly, cells from  
25  
26 cerebella were dispersed with trypsin (0.24 mg/ml; Sigma-Aldrich Italy) and plated at a  
27  
28 density of  $1.5 \times 10^6$  cells/ml on 35 mm Falcon dishes coated with poly-L-lisine (10  $\mu$ g/ml;  
29  
30 Sigma-Aldrich Italy). Granule cells were grown in Basal Eagle's Medium (BME; Celbio  
31  
32 S.p.A., Milano, Italy), supplemented with 10% foetal bovine serum (Celbio S.p.A.), 2 mM  
33  
34 glutamine (Invitrogen s.r.l., Milano, Italy), and 100  $\mu$ g/ml gentamycin (Sigma-Aldrich Italy)  
35  
36 and maintained at 37°C in 5% CO<sub>2</sub>. After 24 hours in vitro the medium was replaced with a  
37  
38 half and half mixture of BME and Neurobasal medium containing 2% B27 supplement, 1%  
39  
40 antibiotic, and 0.25% glutamine. At second day in vitro (DIV2) cytosine arabinofuranoside  
41  
42 (Sigma-Aldrich Italy) was added at the final concentration of 10  $\mu$ M. Recordings were made  
43  
44 from DIV 9-11 neurons in culture.  
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#### 51 52 53 3.2 Solutions and drugs

54  
55 The recording chamber was continuously perfused at 5 ml/min with an extracellular medium  
56  
57 composed of (mM): NaCl 145, KCl 5, CaCl<sub>2</sub> 1, Hepes 5, Glucose 5, Sucrose 20, pH 7.4 with  
58  
59 NaOH all purchased from Sigma-Aldrich Italy. GABA (Sigma-Aldrich Italy) was dissolved in  
60

1  
2  
3 the extracellular solution, ITX in dimethyl sulfoxide (DMSO) at 1 and 10  $\mu$ M, diazepam was  
4  
5 dissolved in DMSO at 10<sup>-2</sup>M and then diluted in the extracellular medium. All drugs were  
6  
7 applied directly by gravity through a Y-tube perfusion system. Drug application had a fast  
8  
9 onset and achieved a complete local perfusion of the recorded cell.  
10  
11

### 12 13 14 15 3.3 Patch clamp recordings

16  
17 Recordings were performed on single neuron after 7-9 days in culture of cerebellar granule  
18  
19 cells at room temperature, under voltage -clamp in the whole-cell configuration of the patch-  
20  
21 clamp technique [Hamill et al. 1981]. Electrodes were pulled from borosilicate glass  
22  
23 (Hidelerberg, FRG) on a vertical puller (PB-7, Narishige) and had a resistance of 5-7 Ohm  
24  
25 when filled with KCl internal solution. Currents were amplified with an Axopatch 1D  
26  
27 amplifier (Axon Instruments, Foster City, California, USA), filtered at 5 kHz, digitised at  
28  
29 10kHz.  
30  
31  
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35

### 36 4 Statistical analysis

37  
38 Data were analyzed using the GraphPad Prism program (GraphPad Software Inc., La Jolla,  
39  
40 California, USA).

41  
42 The behavioral data, expressed as mean  $\pm$  SEM, are obtained by groups of 5 animals each.  
43  
44 One-way analysis of variance (ANOVA) with Dunnett's post-hoc comparisons between  
45  
46 individual treatment groups and vehicle group was performed.  
47  
48

49  
50 Electrophysiological data, expressed as mean  $\pm$  SD, are obtained by 10 cells each group.

51  
52 Control group and diazepam group were compared with the t-test.  
53  
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## Results

As shown in Table 1, ITX was unable to affect animal behavior in the elevated plus maze test at any tested dose. As expected, diazepam significantly ( $p<0.05$ ) increased the total number of entries, the percentage of open arms entries and the percentage of time spent in open arms in comparison with vehicle group, indicating and confirming its anxiolytic effect.

ITX, administered at the doses of 1, 10 and 25 mg/kg, altered neither the total pathway, nor the total number of rearings, as shown in table 2. Diazepam administered at the dose of 2 mg/kg, significantly reduced the total pathway ( $p<0.01$ ) and the number of rearings ( $p<0.05$ ) in comparison to the vehicle group.

The administration of ITX did not affect the latency to the loss of the righting reflex and the sleeping time induced by pentobarbital sodium in comparison with controls (tab.3).

Furthermore ITX did not show the ability to bind the central or the peripheral benzodiazepine receptors, contrary to what diazepam or PK11195 did when used as control drugs, as clearly shown in figure 2 and 3.

Considering the possibility of an allosteric modulatory effect, ITX was challenged for its ability to modify the binding of diazepam to central benzodiazepines receptor (fig. 4) and the result was negative.

Moreover, by using the patch clamp technique in primary cultures of granule neurons we studied the effect of ITX on GABA- evoked currents. ITX applied at 1 and 10  $\mu\text{M}$  did not affect GABA current ( $n=10$ ). In the same experiments diazepam (1 $\mu\text{M}$ ), used as reference drug, was able to potentiate GABA-evoked current of 40% ( $n=10$ ) (fig.5).

## Discussion

There are mounting concern about the impact, sometimes not overt at all, of food contaminants on health and wellness of thousands of people in the world. This topic is particular stressful when one considers the worldwide distribution of food from one country to another with the modern fast transportation system. Hence it seems imperative to prevent massive event of human toxicity to know in advance or as fast as possible the safety of chemical compounds found in food for voluntary or accidental addiction. If the issue is related to infant food such as milk, the issue becomes particularly complex since the growing subjects are more sensible than the adults, especially as far as the impact of contaminants on the CNS development and in turn to behavioral disturbances are concerned. It is clear that for many chemical compounds present in food, we must accept the presence based on the so called ADI (Admissible Daily Intake) which derives from the data obtained after chronic exposure studies and which are in turn converted in the LOAEL (LOWest Adverse Effective Level), an index to calculate the risk and the MOS (Margin Of Safety).

In these last years many examples of contaminants in food from the family of colorants have emerged (i.e. the genotoxic and carcinogenic effect of the colorant Sudan I found in Europe as present in pepper and curry powder). It is imperative, however, to exercise caution before criminalizing some chemical compound present in food. First of all one has to accept traces of contaminants based on the dose-effect demonstration of real health risk. This acceptance is mainly related to the necessity to avoid unnecessary food wasting. Second, if necessary, the claimed potential toxicity of contaminants must be proved with accepted scientific methodology before alerting the public opinion. It is clear that when food contaminants are a real risk for human the law must be severely applied and the contaminant excluded drastically.

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2  
3 In this paper we studied the potential anxiolytic or sedative effect of ITX since this  
4 photoinitiator was found in milk and someone blamed the producers for subtly administering  
5 psychoactive drugs [CODACONS]. According to our results, there was no evidence of ITX  
6 to exert any anxiolytic or sedative effect in rats as demonstrated by pharmacological,  
7 biochemical and electrophysiological experiments. These conclusions are based on the fact  
8 that the major sedative system in the CNS, the GABA receptor complex, is not affected by  
9 relevant doses of ITX.  
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22 Of course the importance of the issue will allow us to extend our studies on this topic for a  
23 better definition in terms of food safety and could avoid such type of accident in the future.  
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Table 1

Effect of ITX on arms exploration in the elevated plus maze test

Treatment (mg/kg)	Arms entries			Time spent in arms	
	Total (n°)	Open (%)	Closed (%)	Open (%)	Closed (%)
Vehicle	7.0±1.6	13.3±9.7	86.7±9.7	3.5±3.3	89.6±3.9
Diazepam 1	15.0±1.1*	42.1±5.8*	57.9±5.8*	19.0±2.2*	69.1±3.1*
ITX 1	3.4±1.4	0.0±0.0	100.0±0.0	0.0±0.0	97.0±2.1
ITX 10	8.4±3.1	13.0±8.3	87.0±8.3	6.2±3.8	86.2±6.1
ITX 25	7.8±2.3	17.7±8.8	82.3±8.8	9.7±7.2	81.2±8.2

The data are expressed as mean±SEM (n=5) of the total number of arms entries and the percentage of open and closed arms entries and of the time spent in open and closed arms in a 5 min test. ANOVA followed by Dunnett's test: \* $p < 0.05$  vs. vehicle.

Table 2

Effect of ITX on locomotor activity of rats in the open field test

Treatment (mg/kg)	Total pathway (cm)	Rearings (n°)
Vehicle	5924±426.2	14.4±1.2
Diazepam 2	2568±975.6**	3.6±1.0*
ITX 1	5230±471.5	14.8±4.8
ITX 10	5139±475.5	10.2±0.9
ITX 25	5283±474.8	11.7±2.3

Values are means±SEM (n=5). ANOVA followed by Dunnett's test: \*\*p<0.01, \*p<0.05 vs. vehicle.

Table 3

Effect of ITX on the latency to the loss of the righting reflex and on the sleeping time induced by pentobarbital sodium in rats

Treatment (mg/kg)	Latency to the loss of the righting reflex (min)	Sleeping time (min)
Vehicle	4.4±0.8	45.2±5.0
ITX 1	4.6±0.5	41.6±2.9
ITX 10	4.6±0.7	44.4±5.3
ITX 25	4.3±0.3	40.3±5.7

All animals received i.p. pentobarbital (35 mg/kg) 1 h after the oral administration of ITX.

The data are expressed as mean±SEM (n=5).

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### Figure Caption

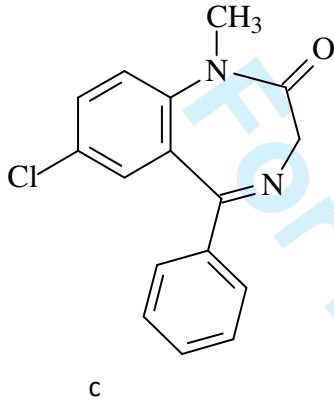
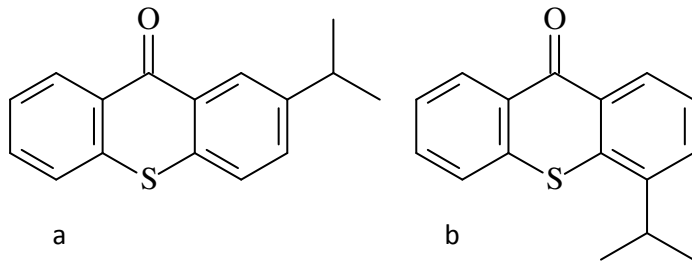
**Fig. 1.** a) 2-isopropyl-9H-thioxanthen-9-one; b) 4-isopropyl-9H-thioxanthen-9-one; c) diazepam

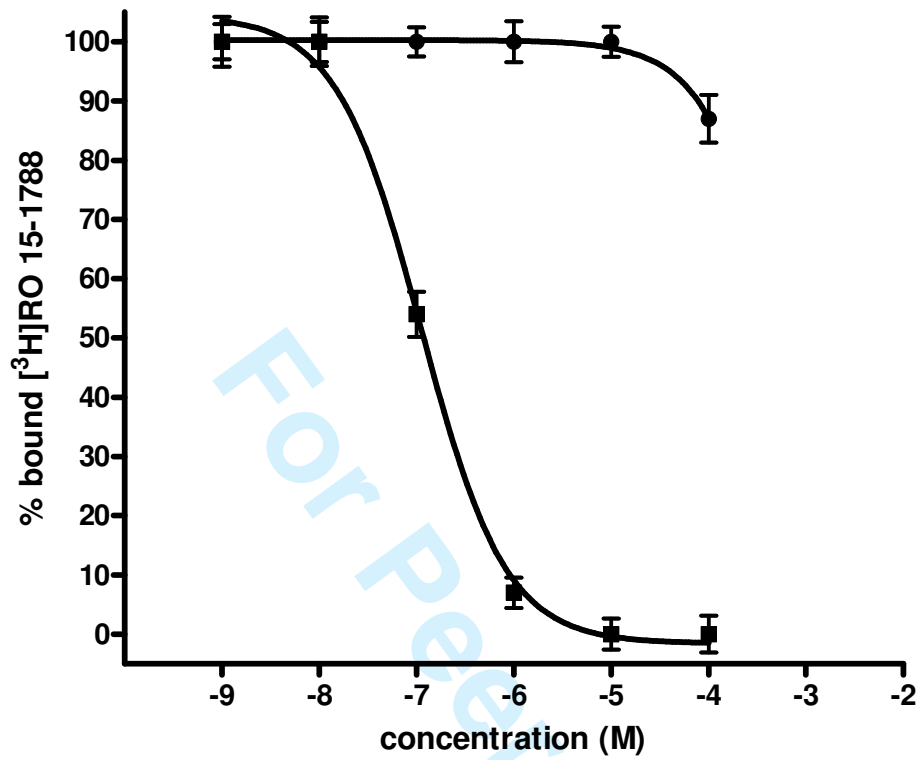
**Fig. 2.** Central BDZ receptor competition binding assay; • ITX-solutions (from  $10^{-4}$  to  $10^{-9}$  M), ▪ diazepam (from  $10^{-4}$  to  $10^{-9}$  M)  $EC_{50}$  0.111  $\mu$ M

**Fig. 3.** Peripheral BDZ receptor competition binding assay; • ITX-solutions (from  $10^{-4}$  to  $10^{-9}$  M), ▪ PK 11195 (from  $10^{-4}$  to  $10^{-9}$  M)  $EC_{50}$  0.199  $\mu$ M

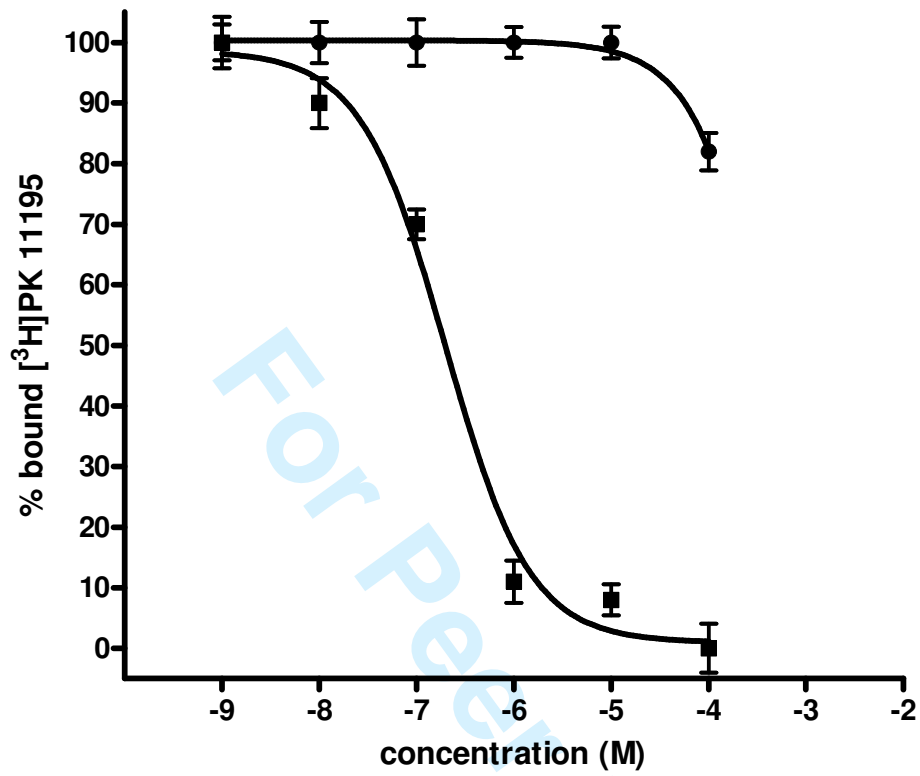
**Fig. 4.** Saturation binding assay; • diazepam added with ITX  $10^{-4}$ M ( $B_{MAX}$  13994 cpm,  $K_D$  1.216 nM), ▪ diazepam without ITX ( $B_{MAX}$  13758 cpm,  $K_D$  1.133 nM)

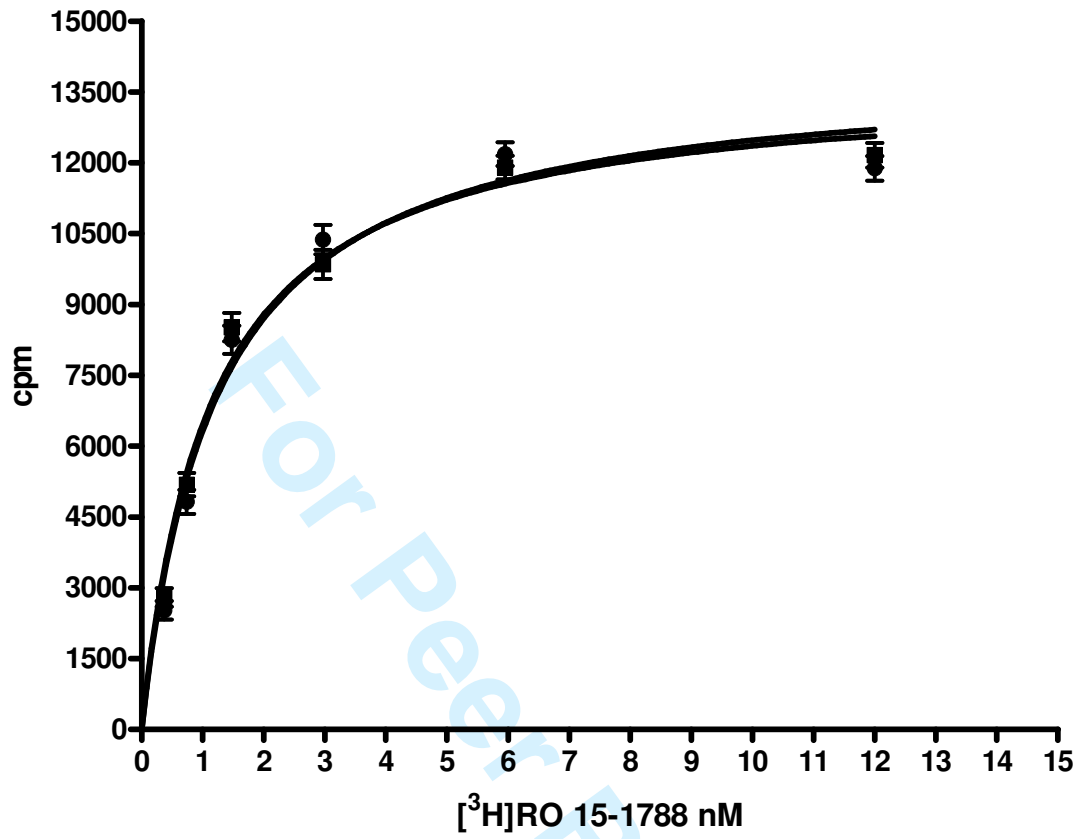
**Fig. 5.** Histograms showing the values (pA) of current evoked by GABA 1 $\mu$ M alone and in presence of ITX (1  $\mu$ M or 10  $\mu$ M) and diazepam 1  $\mu$ M. Each bar is the mean  $\pm$  SD (n=10; \*\*p<0.01, t test)

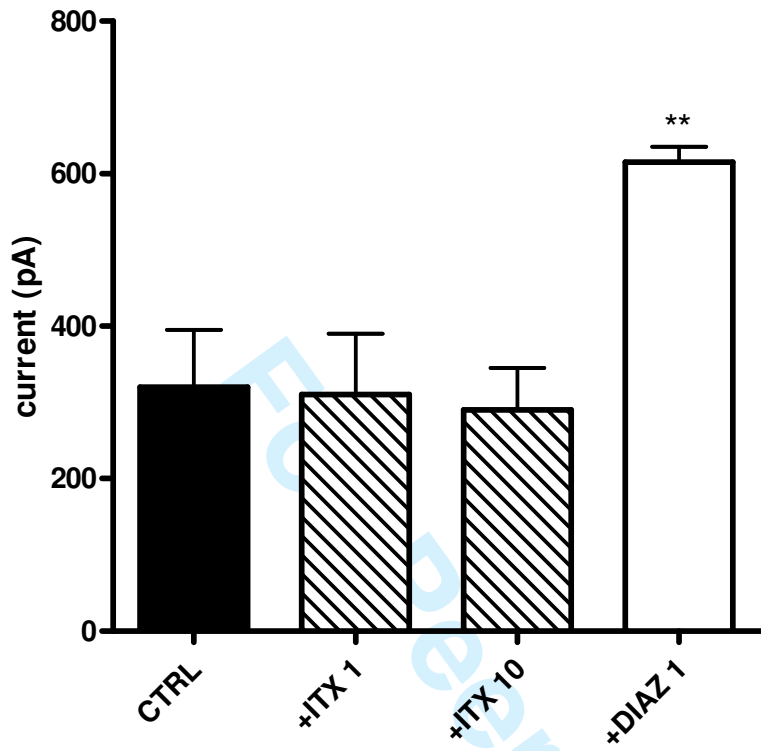












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