On the promoting action of tamoxifen in a model of hepatocarcinogenesis induced by p-dimethylaminoazobenzene in CF1 mice

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Dedicated to the memory of César Polo. Deceased March 9th, 1996.

Abstract

Background and aims: Tamoxifen (TMX) has proven to be an effective palliative treatment for advanced breast cancer with low reported incidence of side effects. TMX has been demonstrated to be an initiator and/or a promoter in the rat model of hepatocarcinogenesis. To document the long-term effect of TMX in mice treated with p-dimethylaminoazobenzene (DAB), we have investigated the time response action of these drugs on different biochemical parameters. Methods: A group of animals was placed on dietary DAB (0.5%, w/w) during a period of 28 weeks. Control animals received a standard laboratory diet. Two other groups of non-treated and DAB-treated animals received TMX citrate (0.025%, w/w) in the diet since day 20. Results: The activities of the enzymes involved in heme synthesis and degradation as evaluated in the DAB group was not further affected by TMX. DAB and/or TMX treatment significantly increased the content of total cytochrome P450 and also the activity of glutathione S-transferase indicating liver damage. In all treated groups oxidative stress and an adaptive response of the natural defense system (catalase and superoxide dismutase) were demonstrated. Histological and morphological studies revealed liver cell hyperplasia in DAB treated group; however, only in the DAB + TMX group solid, trabecular and acinar hepatocellular carcinoma was confirmed at the end of the experimental trial. Conclusion: We have demon

Abbreviations: ALA-S, δ-aminolevulinic acid synthetase; DAB, p-dimethylaminoazobenzene; GST, glutathione S-transferase; HO, microsomal heme oxygenase; P450, cytochrome P450; SOD, superoxide dismutase; TBARS, thiobarbituric reactive species; TMX, tamoxifen.

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strated that TMX produced changes in hepatic enzyme activities which may be relevant for the metabolism and disposition of this and/or other drugs. Because liver tumors could be initiated and promoted by several agents which need to be activated, the possible hazard of TMX should be considered. This study reports that long-term treatment with TMX enhances hepatocarcinogenesis induced by DAB. The widespread use of TMX as an anticancer agent adds to the significance of this study. © 2001 Published by Elsevier Science Ltd.

Keywords: Hepatocarcinogenesis; Tamoxifen; p-Dimethylaminoazobenzene; Cytochrome P450; Oxidative stress

1. Introduction

Tamoxifen (TMX) is a widely used drug with proven efficacy in the treatment of breast cancer [1]. Clinical studies were initiated to determine whether a long-term prophylactic treatment with TMX in women considered to be at high risk of breast cancer, would be beneficial in preventing the onset of the disease [2].

TMX has been shown to cause liver cancer in rats, but not in mouse [3–5]. TMX has been demonstrated to be carcinogenic acting through both genotoxic and non-genotoxic mechanisms [6]. Although this drug is inactive in conventional tests for genotoxicity, it readily forms DNA adducts in rodents liver [7,8], it binds irreversibly to proteins in the presence of liver microsomes [9] and it induces micronucleus formation in human MCL-5 cells [7]. These findings are evidence of genotoxicity and emphasise the importance of elucidating the mechanism by which TMX exerts these deleterious effects. One possibility is the generation of reactive intermediate(s) which would then covalently bind to macromolecules [9].

TMX has the ability to induce P450 [10] stimulating its own metabolism. By this mechanism, TMX could accelerate its elimination and increase the production of reactive genotoxic metabolites [11]. It has been shown that TMX could function as a promoting agent in a two-stage model of hepatocarcinogenesis. TMX increased the number, size and progression of altered hepatic foci as well as the incidence of hepatocellular carcinomas initiated by diethylnitrosamine following partial hepatectomy [10].

To test the hypothesis that TMX might act as a promoter by isomerization to a form having estrogenic activity, the effect of TMX and two of its non-isomerizable fixed-ring analogues was comparatively tested in a model of multistage rat hepatocarcinogenesis. While all three compounds acted as promoters in this model, the potency of TMX was greater than that of the analogues, suggesting that TMX has an intrinsic promoting activity independent of its ability to isomerize to more active estrogenic compounds [10].

It has been reported that TMX is a carcinogen capable of both initiating and promoting liver carcinogenesis in female rats [12,13]. Because the natural history of the hepatic neoplastic disease in mice and humans is similar and both species showed certain resistance to develop liver cancer after TMX administration, we have decided to investigate the effect of TMX in animals receiving a long-term treatment with a chemical carcinogen. Accordingly, we have designed the intoxication protocol to assess the biochemical alterations occurring during the initiation stage of hepatocarcinogenesis [14,15]. To document the long-term effect of TMX in the liver of mice treated with p-dimethylaminoazobenzene (DAB), we have investigated the time response action of these drugs on heme pathway regulation and catalysis, hepatic drug metabolism, the antioxidant defense system and the peroxidation index. Attempts have also been made to correlate changes observed with hepatic injury by means of histological analysis.

2. Material and methods

2.1. Chemicals

Chemicals were reagent grade and were purchased from Sigma (St Louis, MO).
2.2. Animals and treatment

Male CF1 mice weighing 30 g were employed. A group of animals (n = 50) was placed on dietary p-dimethylaminoazobenzene (DAB, 0.5%, w/w) during a period of 28 weeks. Control animals (n = 30) were fed with a standard laboratory diet (Purina 3, Asociación de Cooperativas Argentinas, San Nicolás, Buenos Aires) for the same period. Two other groups of non-treated animals (n = 40) and DAB-treated animals (n = 50), received TMX citrate (Gador Laboratories, 0.025% w/w) in the diet since day 20. All animals received food and water ‘ad libitum’.

Throughout the study all animals were inspected at least twice daily. Body weight and food consumption were measured at intervals throughout the study. Food was removed from animals 16 h before sacrifice. Mice were killed (at least eight animals per group) under ether anaesthesia at the indicated times and liver samples were processed immediately as previously described [16]. All animals received humane care and were treated in accordance with guidelines established by the Animal Care and Use Committee of the Argentine Association of Specialists in Laboratory Animals (AADEALC).

2.3. Assays

δ-Aminolevulinic acid synthetase (ALA-S) activity was measured as described [17] and microsomal heme oxygenase (HO) activity according to Ref. [18].

Cytochrome P450 (P450) content was determined in the microsomal fraction [19].

Glutathione S-transferase (GST) was determined by the method of Ref. [20].

Catalase was measured as described in Ref. [21].

Superoxide dismutase (SOD) activity was determined as described in Ref. [22].

The peroxidation index was evaluated by the formation of malondialdehyde and determined as thiobarbituric reactive species (TBARS) by the method of Ref. [23].

Protein concentration was determined by the method of Ref. [24].

Enzyme units (U) were defined as the amount of enzyme producing 1 nmol of product or consuming 1 nmol of substrate (catalase) under the standard incubation conditions. Specific activity (Sp. Act.) was expressed as U/mg protein. One unit of SOD was defined as the amount of SOD capable of inhibiting by 50% the rate of NADH oxidation measured in the control.

2.4. Histology

At different times (9, 12, 20 and 28 weeks), slices of each of the three main lobes of the liver were fixed in 10% formaldehyde, embedded in paraffin and stained with hematoxylin–eosin, Perl’s Prusian blue to visualize hepatic iron deposits and Masson’s trichrome to determine fibrosis and collagen by using standard techniques. Livers were also obtained at the same times for routine morphological analyses.

2.5. Statistical analysis

Student–Newman–Keuls test was used to assess the degree of significance, using GraphPad InStat V2.00 software.

3. Results

Growth curves as a function of time of treatment with a drug can be indicative of toxicity. We have found a decrease in body weight gained of all treated groups when compared with controls, with lower effects for the TMX group (data not shown). The ratio of liver weight to body weight was significantly increased in DAB and DAB + TMX treated mice, reaching a maximum on week 20 (139 and 106%, respectively), while the ratio for the TMX group was similar to that of the control group (Fig. 1). Although 20–25% of animals receiving DAB died during the course of treatment, the death rates did not differ in groups DAB or DAB + TMX.

As previously reported [14,15], DAB significantly induced ALA-S activity reaching nearly 100% on week 9. However, the inductive effect was 70% in the DAB + TMX group. Then, the
enzyme activity gradually decreased reaching 40% inhibition on week 20. Although TMX alone did not modify ALA-S activity during the first 12 weeks, thereafter the same inhibition profile described for DAB-treated groups was found (Fig. 2A).

Because P450 is involved in TMX activation, its total content in all groups was measured. The levels of P450 in the DAB-treated groups showed a profile similar to that of ALA-S. The high induction (350%) in P450 content produced by DAB [14,15] in week 9, decreased abruptly being only slightly increased in the DAB group and reaching the basal levels in the DAB + TMX group on week 28. P450 content increased 150% on week 9 in animals treated with TMX alone, returning to basal levels between weeks 12 and 20 and then decreased to 40% in week 28 (Fig. 2B).

In all groups, HO activity was significantly diminished (about 40% for DAB groups and 15% for TMX group) when compared with controls on weeks 9 and 12. At longer periods, HO activity showed a progressive induction for animals fed with DAB (Fig. 2C).

As expected [14,15], GST activity, considered as a marker for liver damage, increased about 100% after 20 weeks of DAB treatment, then the enzyme activity diminished on week 28 with a tendency of reaching its lowest values in the
DAB + TMX group. TMX alone produced a 30% enhancement of GST activity between weeks 12 and 20 and a 75% stimulation on week 28 (Fig. 2D).

DAB treatment greatly increased TBARS content [16], reaching its highest values (DAB group: 325%; DAB + TMX: 230%) on week 12 and then remaining unchanged for the rest of the period studied. In the TMX-treated group, TBARS were unmodified during the first 12 weeks and then increased to 60% since week 20 (Fig. 3A).

SOD activity significantly decreased in DAB fed animals [16] during the whole period of the assay, increased in the DAB + TMX group from week 9 onwards and showed a gradual induction to reach its maximum (50%) on week 28 in the TMX group (Fig. 3B).

As expected [16], catalase activity was diminished to 55% by DAB and this diminution persisted during the whole period of the study. This effect was not significantly modified by TMX co-treatment. However, the sole administration of TMX provoked a 30% increase in catalase activity on week 9, afterwards, activity decreased (Fig. 3C).

Histological liver sections of 6/6 DAB treated animals (28 weeks) showed normal architecture with cell hyperplasia, determined by morphological features. The presence of bile thrombosis and abundant hemosiderosis in hepatocytes and in Kupffer cells were also detected (Fig. 4A). Foci of necrosis, irregularly distributed and surrounded by leucocytes, were observed with a frequency of 3/6 (Fig. 4B).

Grossly, at the end point of the assay, hepatic neoplastic soft nodules (0.3–0.4 cm in diameter) at the periphery of the liver left lobe, were observed in four of six of the DAB + TMX treated mice. This pathology was not observed in those mice receiving either DAB or TMX alone.

Microscopically, histological sections of the nodules showed atypical hepatocytes with solid, trabecular and pseudoglandular pattern of growth. Cytologically, the tumor cells were polygonal, had an abundant granular eosinophilic cytoplasm and more than one prominent nucleoli. Neoplastic cells showed intracytoplasmic bile pigment (Fig. 5).

Neighbouring parenchymal cells were dysplastic, with intense cholestasis with intracytoplasmic bile pigment in Kupffer cells and canaliculi, most of the cells showed fatty change microvesicular steatosis (Fig. 6).

These results indicate the carcinogenic potential of chronic TMX administration after DAB initiation in mice liver.

4. Discussion

TMX is a well-tolerated palliative and adjuvant treatment for human breast cancer requiring however, continuous and long-term administration for optimal therapeutic effectiveness [25]. It was suggested earlier [7] that TMX may induce its own
activation in vivo. It has been proposed that TMX exerts its tumorigenic effect by nongenotoxic mechanisms [26]. TMX is a potent liver tumor promoter in the multistage model of rat hepatocarcinogenesis [13] increasing the ratio of liver weight to body weight after subchronic treatment [10], which is suggestive of mild liver hypertrophy masked by overall physical atrophy.

Under our protocol, studies were undertaken for over 6 months during which no variation, when compared with their respective control groups, was detected in the weight ratio in CF1
mice, either in animals treated with TMX alone or in those receiving DAB + TMX, reflecting that TMX by itself would not evoke any additional liver damage.

We have found here that the activities of the enzymes involved in heme synthesis and degradation as evaluated in the DAB group were not further affected by TMX. However, adaptive compensatory responses seem to be the most likely explanation for the majority of the changes observed after TMX administration in the drug metabolizing system and in the oxidation status. TMX is a mixed inducer of phase I enzymes with a weak phenobarbital-like pattern of induction [27]. TMX also has a significant effect on hepatic phase II enzymes expression, which may have implications for the carcinogenicity and/or therapeutic activity of the drug [28].

We have demonstrated that TMX by itself significantly increased the content of total P450 on week 9 but then produced a noticeable diminution on week 28. Specific cytochromes P450 have been associated with TMX metabolism [27]. Further studies measuring P450 levels using specific antibodies are in progress. It is worth noting the great increase in GST activity towards the end of the assay, reflecting liver damage. This could be of particular importance, since TMX requires metabolic activation for the subsequent formation of covalently bound protein adducts [9]. It has been suggested [29] that a faster rate of metabolism and the production of polar metabolites may indicate the ability of mouse to detoxify TMX by its rapid elimination, compared with rat and human. In our experiments, we have shown a great increase in SOD activity that could be ascribed to the production of free radicals and the need to enhance the natural defense system.

Other properties of TMX may cause alterations in liver cell homeostasis and thereby lead to liver damage. The effects of antiestrogens, like TMX, inducing P450-mediated monooxygenase activity, on oxidative stress, remain to be clarified.

The diminution of catalase and SOD activities in DAB-treated animals might be the result of their sensibility to at least one of the ROS generated under oxidative stress. Inhibition of both enzymes could be the consequence of an irre-

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Fig. 5. Histological features of liver nodules. Mice were treated with DAB (0.5% w/w) and TMX (0.025% w/w) for 28 weeks. Liver was sectioned and stained with hematoxylin–eosin (magnification × 160). The figure shows hepatocellular carcinoma solid, trabecular and acinar type. Other experimental conditions are as indicated in Section 2.
Fig. 6. Histological features of liver surrounding nodules. Mice were treated with DAB (0.5% w/w) and TMX (0.025% w/w) for 28 weeks. Liver was sectioned and stained with hematoxylin–eosin (magnification ×160). The figure shows parenchyma cells were dysplasic, with intense cholestasis and microvesicular steatosis. Other experimental conditions are as indicated in Section 2.

versible autocatalytic process, in which the sustained increase of ROS would finally lead to cellular death [30].

Thus, it is possible that TMX, acting as a mixed agonist/antagonist of the estrogens receptor, alters the hormonal milieu of the animal, thus facilitating and/or antagonizing xenobiotic metabolizing enzyme induction. Since TMX provokes changes in hepatic enzyme activities in mice, enzyme induction may also occur in humans and this may be relevant for the metabolism and disposition of this and of any other drug [31]. Because liver tumors could be initiated and promoted by several agents, which need to be activated, the possible hazard of TMX should therefore be considered.

Histological studies have confirmed the presence of solid, trabecular and acinar carcinoma at the end of the experimental trial (28 weeks) in the animals treated with DAB + TMX with a frequency of 4/6.

The present study demonstrates that long-term treatment with TMX promotes hepatocarcinogenesis initiated with DAB in mice. It is worth noting that the same dose in rat increases the incidence of hepatocellular carcinoma in diethylnitrosamine-initiated lesions [6] and that this dose is comparable to the levels observed in patients under usual treatment protocols [10]. In contrast to previously published results with our model, we have found that TMX fails to initiate hepatocarcinogenesis. This apparent disagreement may be due to the use of different animal species and the length of treatment [5,31]. For agents such as TMX that are DNA-reactive, it is not possible to quantify a promoting effect, because when a DNA-reactive carcinogen is given after another it can produce a syncarcinogenic effect which is a consequence of summing up DNA damage [32].

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