Comparison of the effects of tamoxifen and toremifene on rat hepatocarcinogenesis

Received: 29 December 1999 / Accepted: 24 March 2000 / Published online: 11 May 2000
© Springer-Verlag 2000

Abstract The hepatoproliferative and cytochrome P450 enzyme inducing effects of two antiestrogens, tamoxifen and toremifene, were compared in female Sprague-Dawley rats using immunohistochemical staining methods. Equimolar doses of the antiestrogens (tamoxifen 45 mg/kg and toremifene 48 mg/kg) were given by oral administration to 6-week-old rats for 12 months including a 3-month recovery period. Controls received the vehicle carboxymethylcellulose. Altogether 90 rats were used in the study. Five rats per dose group were killed after 14 days, 5 weeks, 3, 6 and 12 months of treatment as well as after the 3-month recovery period. Hepatocellular carcinoma was found in four out of five rats after 12 months of tamoxifen treatment. After the 3-month recovery period all tamoxifen-treated rats had large liver tumors (diameter up to 3 cm). No tumors were observed in toremifene-treated rats. Liver cell proliferation was measured by the index of proliferating cell nuclear antigen (PCNA) expression. Immunohistochemical staining with the placental form of glutathione S-transferase (GST-P) was used as a marker for preneoplastic foci. Cytochrome P450 induction was measured using specific antibodies to isoenzymes. Tamoxifen increased the incidence of GST-P-positive foci significantly by 3 months of treatment but toremifene did not as compared with the controls. Liver cell proliferation increased significantly only in the liver tumors of tamoxifen-treated rats after 12 months of treatment and during the recovery period. Both antiestrogens induced the isoenzymes CYP2B1/2 and CYP3A1 within 14 days although tamoxifen was a more powerful inducer. Immunohistochemistry of rat liver sections showed a centrilobular localization of these induced enzyme proteins. The expression of CYP2B1/2 and 3A1 could also be observed in foci after 3 and 6 months of administration and in liver adenomas and in some carcinomas after 12 months of administration with tamoxifen. The results show that tamoxifen, but not toremifene, has the potential to induce and promote the development of rat hepatocarcinogenesis in this experimental model.

Key words Antiestrogens · Tamoxifen · Toremifene · Hepatocellular carcinoma · Immunohistochemistry · Enzyme induction

Introduction

Tamoxifen is a nonsteroidal antiestrogen which has been used in the therapy of advanced breast cancer since the 1970s (Jordan 1992). Preventive trials have been initiated in which tamoxifen is given to healthy women at high risk of developing breast cancer (Fugh-Berman and Epstein 1992; Vanchieri 1992). However, the safety of tamoxifen in long-term therapy has been questioned (Seachrist 1994; Marshall 1994). In recent years evidence has been accumulated that tamoxifen is a genotoxic hepatocarcinogen in the rat (Greaves et al. 1993; Hard et al. 1993). Tamoxifen has been shown to increase the incidence of liver tumors in the rat in a time- and dose-dependent manner (Hard et al. 1993; Hirsimäki et al. 1993; Williams et al. 1993). Tamoxifen is known to be capable for both initiation (Dragan et al. 1994a; Williams et al. 1997; Carthew et al. 1995b) and promotion of cancer in the livers of several strains of rat (Carthew et al. 1995a, b). In humans, long-term therapy with tamoxifen is associated with an increased risk of endometrial cancer (Rutqvist et al. 1995; Fornander et al.)
1989). IARC (The International Agency for Research on Cancer) has stated that tamoxifen apparently increases the risk of endometrial cancer in humans although the benefits of tamoxifen in the treatment of patients with breast cancer exceed the associated risks (IARC Monographs 1996). Tamoxifen is not known to cause liver cancer in humans.

The antiestrogen toremifene is a closely related analog of tamoxifen, which differs from tamoxifen by the presence of a chlorine atom in the ethyl side chain (Fig. 1) (Sipilä et al. 1990). Toremifene has similar antiestrogenic and estrogenic properties to tamoxifen (Kendall and Rose 1992; Kangas 1990). However, toremifene has not been shown to form significant DNA adducts in rat liver (Hard et al. 1993; White et al. 1992) and does not induce liver tumors in rodents (Hard et al. 1993; Hirsimäki et al. 1993). Toremifene has not shown to be either genotoxic or carcinogenic to humans.

Hepatic cytochrome P450-dependent monooxygenases are known to be responsible for much of the metabolism of various xenobiotic chemicals. Tamoxifen needs to undergo metabolic activation to reactive species in order to exert its genotoxic and carcinogenic effects (White et al. 1992; Pathak et al. 1995; Pathak and Bodell 1994; Pongracz et al. 1995; Li et al. 1997; Styles et al. 1994; Sargent et al. 1994). Tamoxifen treatment of rats yields hepatic DNA adducts (Li et al. 1997) which may be critical, since there is an apparent correlation between the formation of DNA adducts and the subsequent formation of tumors (Carthew et al. 1995a, b; White et al. 1992; Montandon and Williams 1994).

The aim of the present study was to clarify the mechanisms which operate at the beginning of carcinogenesis and which regulate multistage hepatocarcinogenesis. We have investigated the effects of antiestrogens on the appearance of glutathione S-transferase P (GST-P)-positive foci. Expression of GST-P-positive foci is an indicator of preneoplasia in liver carcinogenesis (Kithara et al. 1984). Liver cell proliferation is an important factor in the carcinogenic process. By determining the proliferating cell nuclear antigen (PCNA) index, we have compared the effects of tamoxifen and toremifene on hepatocyte proliferation. Since induction of liver cytochrome P450 activities by xenobiotics may have an important implication for metabolic activation of the antiestrogens, we localized the cytochrome P450 isoenzymes which might be involved in their metabolism.

**Materials and methods**

**Animals**

Six-week-old female Sprague-Dawley rats were used in the experiment. The rats were purchased from Møllegaard Aps (Denmark). The animals were fed with a standard diet (R3, Astra-Ewos, Sweden) and given tap water ad libitum. The animals were kept in controlled relative humidity (50 ± 20%) and temperature (20 ± 2°C) and a 12–12 h controlled light-dark rhythm (lights on 0600–1800 hours). There were approximately 15 air changes/h in the animal room. The rats were weighed regularly during the experimental period. The studies were performed following good laboratory practice (GLP).

**Treatment**

More than 99% pure Z-isomers of tamoxifen and toremifene were synthesized as citrate salts in the Chemical Research Laboratory of Orion Corporation (Medipolar, Oulu, Finland). Equimolar dose levels of tamoxifen citrate and toremifene citrate were used: tamoxifen 45 mg/kg and toremifene 48 mg/kg. The high doses used were the maximum tolerated doses for Sprague-Dawley rats in long-term experiments. The vehicle, carboxymethylcellulose (CMC, 0.5%), was given to control animals. Dosing was performed by oral gavage using a stainless steel cannula on 7 days/week for 12 months. Five rats per dose group were killed after 5 weeks, 3, 6 and 12 months of dosing. A 3-month recovery group after 12 months of dosing was also included. There was also a further group of rats, the dosing of which lasted for only 14 days. Altogether 90 rats were used in the study.

**Autopsy and histopathology**

At the interim kills and at the end of the study, the animals were asphyxiated with CO₂ and specimens from liver lobes and of all liver lesions observed at autopsy were taken for light-microscopic examination, fixed in 10% buffered formalin and embedded in paraflin. Tissue sections were stained with Delafield’s hematoxylin eosin.

**Immunohistochemical stainings**

Four-micrometer-thick sections were cut from formalin-fixed paraflin-embedded liver samples from the left lateral lobe of the liver and also from the macroscopically visible liver tumors of the tamoxifen-treated rats. Immunohistochemical stainings were performed using a TechMate 500 immunostaining machine and a peroxidase/diaminobenzidine (DAB) multilink detection kit (DAKO, Denmark) which is based on an indirect streptavidin biotin method. Sections were counterstained with Mayer’s hematoxylin.

**Immunohistochemical detection of GST-P**

GST-P-positive foci were detected from livers at 5 weeks, 3, 6, 12 and 12 + 3 months time points. Paraflin sections were rehydrated and stained for GST-P proteins by using an anti-GST-P polyclonal antibody (1:2000 dilution, Pan Vera Co., Madison, USA). Single GST-P foci (defined as groups of five or more cells) were counted from liver sections from animals at each time point. Adenomas and carcinomas in tamoxifen-treated rats at time points 12 months and