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Tamoxifen administration and metabolism in nude mice and nude rats☆

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Abstract

We investigated the kinetics of tamoxifen (tam) in immunodeficient mice and rats after oral treatment and compared drug and metabolite profile in nude rat serum and tissues after oral and subcutaneous (s.c.) routes of administration. The serum levels were compared to those observed in man. After oral dosing in mice, tam and the potent metabolite 4-hydroxytamoxifen (4-hydroxytam), were detectable in liver and lung tissue, but not in serum. The levels of 4-hydroxytam in these tissues were significantly higher than those of tam, a profile opposite to that observed in rat and man. In rats and man, the 4-hydroxytam/tam serum concentration ratios were 0.16 and 0.02, respectively. Compared to oral route, the s.c. pellets yielded only trace amounts of the demethylated derivatives of tam in rats. Thus, the kinetics of tam observed in the present study suggest that the nude rat may represent a preferable animal model in studying the pharmacokinetics of tam and that, the oral route yielded higher serum and tissue levels of tam and metabolites than equivalent s.c. pellet implants.

Keywords: Tamoxifen; Pharmacokinetics; Nude rats; Nude mice; Metabolism

1. Introduction

The antioestrogen tam is a first line drug in the treatment of breast cancer. It was first approved in the UK in 1973 and by the Food and Drug Administration (FDA) in the US in 1977, for the treatment of advanced breast cancer. In 1998, it was approved for the reduction of breast cancer risk in high-risk women in the US [1]. However, the drug has some serious side effects, such as endometrial cancer [2] and thromboembolic diseases [3,4] that limit its use in healthy women.

No proper dose finding studies have been performed for tam. Serum concentrations of the drug and its metabolites may vary with age [5], menopausal status [6], and body mass index [7]. The importance of these factors on the effect of tam treatment remains to be elucidated. In addition, it is not known whether doses needed for prevention are identical to those needed for breast cancer therapy [5,8,9].

Multiple studies on tam for treatment and prevention of breast cancer have been performed in rodents. In mice, the potent metabolite 4-hydroxytam is present in high concentrations [10,11], whereas its concentration in rats and man is low. Thus, a rat model may be the preferable animal model for studying the pharmacokinetics of tam.

Human transplants in immunodeficient (nude) rodents represent a model for in vivo studies of drug effects on human tissues [12]. However, pharmacokinetics and responses to drugs may vary even between different strains of the same rodent species [5]. The tam undergoes first pass metabolism that affects its oral bioavailability, but the routes of administration used in animal studies, i.e. oral [13], intraperitoneal injection [14] subcutaneous (s.c.) injections or s.c. implants [11,15] may modify the end results. The aims of our study were to compare tam metabolism in nude rats and nude mice, as well as two routes of tam administration (oral versus s.c.), in nude rats.

2. Material and methods

2.1. Drugs and chemicals

The tam citrate (minimum purity 99%) and "HPLC grade" diethylamine were purchased from Sigma–Aldrich (Steinheim, Germany). The vehicle, 1,2-propandiol, was obtained from Merck–Schuchardt (Hohenbrunn, Germany). The 50 mg 60-day continuous release tam pellets and the 0.18 mg 60-day continuous release 17β-oestradiol pellets were purchased from The Innovative Research of America (Sarasota, FL). Throughout the experiments, tam was

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dissolved in the vehicle (2 or 12 mg/ml) and administered orally at 2, 5 or 40 mg/kg-body weight.

The tam, *N*-desmethyltamoxifen (*N*-desmethyltam) and *N*-desdimethyltamoxifen (*N*-desdimethyltam) standards with purity >98%, were gifts from Imperial Chemical Industries, PLC Pharmaceuticals division (Macclesfield, UK). The "HPLC grade" acetonitrile was purchased from Merck KgaA (Darmstadt, Germany) and acetic acid (HiperSolv) was obtained from BDH laboratory supplies (Poole, England).

2.2. Animals and man

Rowett nu/nu female rats weighing 130–160 g during experiments and female NCR athymic nude mice weighing 25–33 g were bred in the Tumour Biology Department, Norwegian Radium Hospital (Oslo, Norway). Animals were housed in grid-bottom metal wire cages in a temperature controlled (22±3 °C) room with 12 h light/dark cycles. They were acclimatised for 14 days before start of experiments and had free access to water and a standard diet from Beekay Feeds, B & K Universal AS (Nittedal, Norway).

Four-week-old nude rats and nude mice were oophorectomized 7 days before the start of tam administration under isoflurane inhalation anaesthesia (Abbott Scandinavia AB Solna, Sverige). A single 0.18 mg 60-day sustained release 17β-oestradiol pellet was implanted subcutaneously (s.c.) and all animals were kept in different cages (one animal per cage). The oestradiol pellet was implanted in order to standardise oestrogen levels because breast cancer growth is usually oestrogen dependent. This is in accordance with previous studies in rodents bearing breast cancer xenografts [16,17].

The study consisted of the following sets of experiments:

(1) *Comparison of nude mice versus nude rats*: Nude mice were divided in three groups. The first ($n = 8$) received tam 2 mg/kg per day, the second (n=8) 40 mg/kg per day and the third ($n = 4$) received vehicle only. Nude rats were treated in an identical experiment. They were sacrificed on Day 5.

(2) *Comparison of oral treatment versus s.c. pellets in nude rats*: The rats were divided in three groups, the first group ($n = 5$) received 5 mg/kg per day orally, in the second group ($n = 5$), a 50 mg 60-day continuous release pellet was implanted s.c., and the controls ($n = 4$) received vehicle only. The three groups were sacrificed on Day 21. The animals were sacrificed under carbon dioxide gas 24 h after the last dose.

All experiments were approved by The Norwegian Animal Research Authority and conducted according to the European Convention for the Protection of Vertebrates used for scientific purposes.

(3) *Breast cancer patients*: Blood samples were collected from six breast cancer patients who were on a daily tam dose of 30 mg once a day for 56 days. The steady-state samples were collected 24 h after the last dose. Three patients were premenopausal (age range 35–45 years) and three were postmenopausal (age range 51–74 years). Three had metastatic disease and three received adjuvant tam treatment.

2.3. Sampling

2.3.1. Serum and tissue samples

Tissue samples from liver, lung, retroperitoneal adipose tissue, kidneys, brain, muscle, heart, spleen and uterus were dissected. They were immediately frozen in liquid nitrogen and stored at −80 °C until analysis. Blood samples (0.9 ml) were drawn from the tail vein of the nude rats on Days 7, 14 and 21. Blood was allowed to clot at room temperature for 1 h before centrifugation at 3000 × *g* for 15 min. The serum obtained was also stored at −80 °C until analysis.

2.4. Sample processing

Serum was routinely processed by centrifugation of a mixture containing an equal volume of the sample and 100% acetonitrile, at 15,000 × *g* for 6 min. The supernatant was transferred to sample vials for analysis. Tissue samples weighing about 0.4 g were homogenised (1:5, (w/v)) at 26,000 rev/min in ice-cold 50 mM Tris–HCl buffer, pH 7.4, using a Polytron PT-MR 2100 homogeniser from Kinematica AG (Luzern, Switzerland). The homogenates were mixed with an equal volume of 100% acetonitrile and the precipitated proteins were removed by centrifugation at 15,000 × *g* for 6 min. The supernatants were transferred to sample vials for analysis.

2.5. Instrumentation

2.5.1. High performance liquid chromatography

Serum and tissue concentrations of tam and its metabolites were measured using methods previously described [18,19]. Briefly, tam and its metabolites were determined in an acetonitrile extract from serum or homogenised tissues and were separated by reverse-phase, low-dispersion liquid chromatography. A fluorescence detector detected tam and its metabolites after an on-line conversion to fluorophors by UV light. The within-day precision for tam and the metabolites measured were 0.7–5.6% for concentrations between 10 and 800 ng/ml. The detection limits were 1 ng/ml and the recovery from human serum ranged from 100 to 108% whereas recovery from rat tissues ranged from 73 to 103%. In mice tissues the recovery of tam, 4-hydroxytam and *N*-desmethyltam was above 69.4%.

The assay was modified to improve the separation and sensitivity of the highly potent metabolite, 4-hydroxytam. The pre-column length was decreased from 30 to 8 mm and the emission wavelength was changed from 360 to 383 nm.

3. Results

3.1. Comparison nude mice versus nude rats

In contrast to rats, tam and metabolite concentrations in mice treated with 2 mg/kg per day of tam were not detectable. All studied rat tissues had higher tam and metabolite levels than the corresponding mice tissues.

At 2 mg/kg per day oral tam, results indicated that tissue and serum concentrations of tam and its metabolites were different between these two species and that metabolite profile in nude rats, compared to that in mice, was more like the situation in man. We, therefore, performed the second experiment only in nude rats. The objective was to determine the influence of two frequently used routes of administration on the kinetics of tam.

Mice treated with 40 mg/kg per day of tam had detectable levels in the liver and lung tissues, and the levels of 4-hydroxytam were significantly higher than those of tam, a profile opposite to what is normally seen in man (Table 1). In tissue samples of rats treated with 40 mg/kg per day, the levels of 4-hydroxytam were below 20% of tam's levels. This is a profile similar to the situation in man.

3.2. Oral versus subcutaneous dosing in nude rats

According to data from the manufacturer, the engineered Matrix-Driven Delivery (MDD) pellet system integrates the three principles of diffusion, erosion and concentration gradients. The finished pellet contains a biodegradable matrix whose tam elimination should be uniform over time. The 50 mg 60-day release tam pellet is constructed to deliver about 0.833 mg of tam daily, which would approximate a total of 17.5 mg of tam in 21 days. Thus, assuming 100% absorption of tam after oral dosing, the two regimens would have delivered comparable amounts of tam to the animals.

After chronic oral dosing of tam in nude rats for 21 days, a rapid increase in serum tam levels was observed. The serum concentrations of tam on Days 7, 14 and 21 were twice as high in the oral group as in the group with s.c. implantation of tam pellets, and the levels of 4-hydroxytam were approximately three–four times higher in the oral group throughout the experimental time (Fig. 1). The serum 4-hydroxytam/tam ratio in nude rats after 7, 14 and 21 days of oral tam dosing were: 0.27 ± 0.05, 0.24 ± 0.05 and 0.38 ± 0.04, respectively, and after s.c. dosing were: 0.13 ± 0.03, 0.17 ± 0.06 and 0.27 ± 0.06, respectively. Another significant difference observed between the oral and s.c. treatments was the absence of detectable amounts of demethylated metabolites in the s.c. group throughout the experimental time (Fig. 1). A representative chromatogram is depicted in Fig. 2.

In contrast to the levels observed in man, the concentrations of *N*-desmethyltam during the first 2 weeks of oral treatment were below those of tam in rats (Fig. 1). However, in the samples collected on Day 21, *N*-desmethyltam concentrations were higher than those of tam. The 4-hydroxytam

Table 1
Concentrations of tamoxifen and metabolites, and the metabolite/parent drug ratios in nude mice and nude rats after oral daily dosing[a] of tamoxifen for 5 days

	tam[b] (ng/g)	4OHtam (ng/g)	*N*Dtam (ng/g)	4OHtam/tam	*N*Dtam/tam
Nude mice (40 mg/kg per day)					
Serum	ND[c]	ND	ND	–	–
Liver	146 ± 43	307 ± 90	48 ± 17	2.1 ± 0.18	0.32 ± 0.07
Lung	128 ± 61	245 ± 69	172 ± 47	1.9 ± 0.49	1.30 ± 0.20
Brain	ND	ND	ND	–	–
Adipose	ND	ND	ND	–	–
Nude rats (2 mg/kg per day)					
Serum	100 ± 29	ND	55 ± 11	–	0.55 ± 0.12
Liver	581 ± 281	301 ± 202	382 ± 184	0.52 ± 0.12	0.66 ± 0.10
Lung	473 ± 277	472 ± 148	816 ± 230	1.0 ± 0.01	1.70 ± 0.38
Brain	35 ± 16	ND	27 ± 11	–	0.77 ± 0.16
Adipose	ND	ND	ND	–	–
Nude rats (40 mg/kg per day)					
Serum	241 ± 101	40 ± 10	187 ± 79	0.16 ± 0.14	0.77 ± 0.12
Liver	4988 ± 1660	1415 ± 390	7186 ± 2171	0.28 ± 0.11	1.40 ± 0.27
Lung	15485 ± 5935	7237 ± 2104	18010 ± 5865	0.48 ± 0.07	1.20 ± 0.19
Brain	1145 ± 238	113 ± 16	1516 ± 314	0.10 ± 0.01	1.35 ± 0.29
Adipose	2863 ± 2140	41 ± 13	288 ± 192	0.01 ± 0.008	0.10 ± 0.08
Man[d]					
Serum	141 ± 49	3.5 ± 1.1	220 ± 57	0.02 ± 0.01	1.62 ± 0.28

Data are presented as mean ± standard deviation ($n = 4$–8).
[a] Sampling was performed 24 h after last dose. Data not corrected for recovery.
[b] tam: tamoxifen; 4OHtam: 4-hydroxytamoxifen; and *N*Dtam: *N*-desmethyltamoxifen.
[c] Not detected.
[d] 30 mg per day for 56 days.

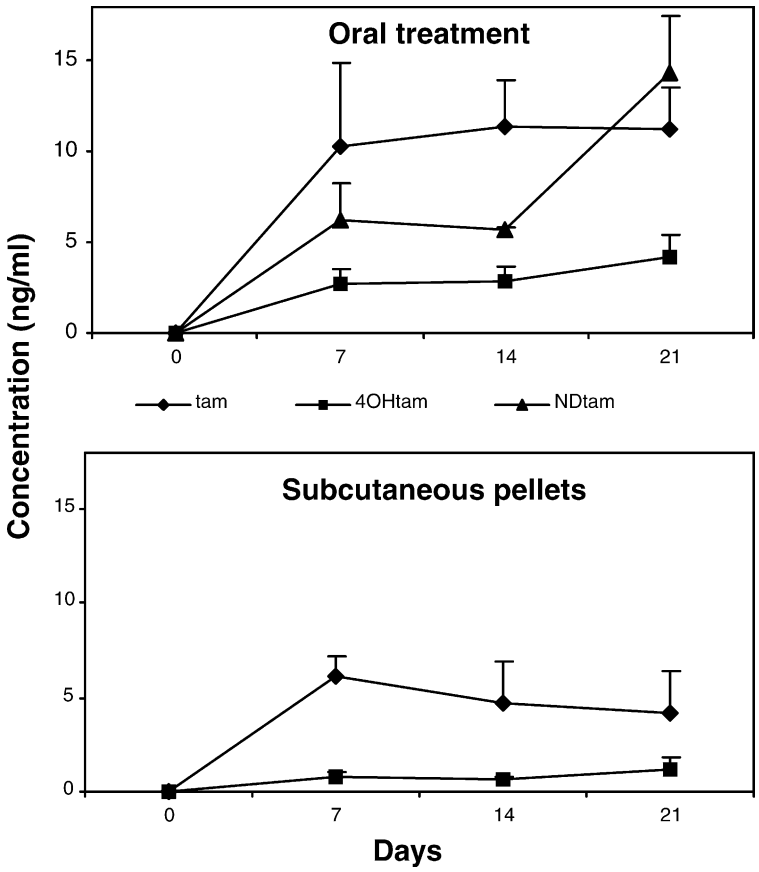

Fig. 1. Concentration curves for tamoxifen (tam), 4-hydroxytamoxifen (4OHtam), and *N*-desmethyltamoxifen (*N*Dtam) in serum during 21 days of oral treatment with 5 mg/kg per day tam or s.c. implantation of 50 mg 60-day continuous release tamoxifen pellets in nude rats. Data presented as mean ± standard deviation ($n = 4$–5).

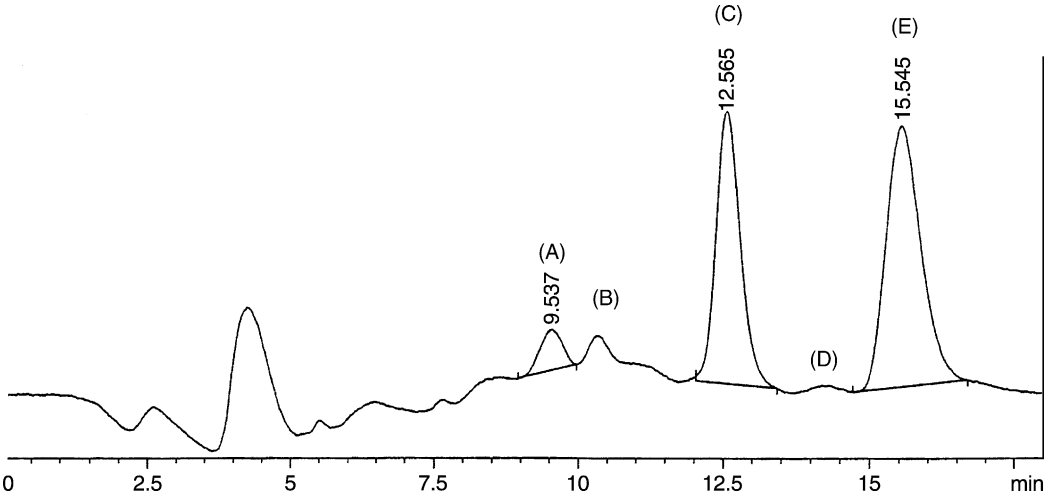

Fig. 2. A chromatogram of an extract from nude rats treated orally with 40 mg/kg per day tam for 5 days. Samples were prepared and subjected through a reverse-phase column as described in the text. (A) 4-Hydroxytamoxifen; (B) unidentified metabolite; (C) tamoxifen, (D) *N*-desdimethyltamoxifen; and (E) *N*-desmethyltamoxifen.

Table 2
Tamoxifen and metabolite concentrations in serum and tissues[a] of nude rats after 21 days of oral treatment with tamoxifen 5 mg/kg per day and by subcutaneous implantation of 50 mg continuos release tamoxifen pellets

	tam[b] (ng/g)		Ratio[c]	4OHtam (ng/g)		Ratio	*N*Dtam (ng/g)		Ratio
	Oral	s.c.[d]		Oral	s.c.		Oral	s.c.	
Serum	11 ± 2	4 ± 2	2.7	4 ± 1	1 ± 0.7	3.7	14 ± 3	ND[e]	–
Liver	478 ± 50	295 ± 42	2.7	280 ± 43	93 ± 5	3.0	487 ± 83	34 ± 1	19.7
Lung	844 ± 510	226 ± 42	3.2	491 ± 253	55 ± 11	6.5	2438 ± 1284	102 ± 17	20.2
Brain	150 ± 27	59 ± 7	3.6	29 ± 3	9 ± 4	7.1	269 ± 29	17 ± 3	15.8
Adipose	1934 ± 586	213 ± 108	9.1	60 ± 14	15 ± 13	3.9	330 ± 185	16 ± 9	20.9
Heart	119 ± 18	44 ± 17	9.6	47 ± 5	2 ± 2	5.6	247 ± 40	13 ± 3	18.3
Spleen	551 ± 112	173 ± 23	3.2	158 ± 45	24 ± 4	8.7	1125 ± 278	56 ± 11	20.6
Muscle	65 ± 17	18 ± 7	1.6	29 ± 4	4 ± 0	3.0	113 ± 24	7 ± 3	14.2
Uterus	494 ± 134	52 ± 22	3.7	48 ± 17	8 ± 3	9.0	213 ± 50	12 ± 1	24.0
Kidney	367 ± 33	115 ± 18	2.5	121 ± 22	14 ± 2	3.4	713 ± 92	35 ± 7	15.4

Data are presented as mean ± standard deviation ($n = 4$–5).
[a] Sampling was performed 24 h after last dose.
[b] tam: tamoxifen; 4OHtam: 4-hydroxytamoxifen; and *N*Dtam: *N*-desmethyltamoxifen.
[c] Ratio of concentrations after oral and s.c. dosing.
[d] s.c.: subcutaneous implantation of pellets.
[e] Not detected.

levels ranged between 1.5 and 5.5 ng/ml and were similar to those usually observed in man after chronic dosing (Table 1).

After 21 days of oral treatment, the highest concentrations of tam were observed in the lungs and adipose tissues of the orally treated group, and in the liver, lung and adipose tissues of the s.c. treated group (Table 2). The ratios (metabolite/parent drug) were independent of route of administration in the different tissues. However, the oral group had generally higher levels of almost all compounds examined compared to the corresponding tissues in the s.c. group (Table 2). Of the nine studied tissues, *N*-desdimethyltam was detected in all tissues of the oral group, whereas minor amounts were detected in only three tissues (adipose, lung, spleen) of the s.c. group (data not shown).

4. Discussion

In this report, we have studied the biotransformation of tam in nude mice and nude rats and the two frequently used routes of administration.

The 4-hydroxytam levels in nude rats were, as in man, below those of tam, whereas in mice 4-hydroxytam was present in higher concentrations than tam. Thus, our results demonstrate major differences in the biotransformation of tam between nude rats and nude mice. This is consistent with previous reports on normal rat and mice strains [13], and suggests that, though actual drug concentrations may differ among the three species (mice, rats, man), the 4-hydroxytam/tam ratio in mice is reverse to that observed in rats and man [11,13,20]. Since 4-hydroxytam has been reported to be about 100 times more anti-oestrogenic than tam [21,22], this observation is of importance when selecting laboratory animals for studying tam effects on human transplants.

Given 100% tam absorption after oral dosing, the two routes of administration would have resulted in identical total drug dose during 21 days of treatment. However, the 50 mg slow release pellets produced lower concentrations of tam and 4-hydroxytam compared to the oral treatment, and in addition, *N*-desmethyltam and *N*-desdimethyltam were not detectable in serum.

The observation that serum levels of *N*-desmethyltam had a second increase in the third week of treatment was a surprise. Our previous studies with male and female Wistar rats indicated identical profile and concentrations of tam and metabolites in serum and tissues after 3 and 14 days of a chronic oral tam dose of 1 mg/kg per day. It was therefore suggested that steady state conditions were obtained already after three days of treatment [19].

The second increase in *N*-desmethyltam levels may be due to interactions with cytochrome P450 (CYP) enzymes because tam is an inducer of CYP 3A in rats [23] and human hepatocytes [24]. This enzyme converts tam to *N*-desmethyltam [23,25,26] and the observed induction in rats was significant after two weeks of tam treatment [23,25,26]. The increase in *N*-desmethyltam observed in this study was not reflected in other compounds. This may suggest that the excretion of 4-hydroxytam is faster than that of *N*-desmethyltam. This is in keeping with the observation that conjugates of hydroxylated metabolites are the dominating excretory compounds in man and may indicate that conjugation followed by hydroxylation could be the main excretory pathway also in rats [27].

Robinson et al. exposed Sprague–Dawley rats to oral doses of tam (200 mg/kg per day) in peanut oil [13]. As their

study did not exceed 7 days, they were not able to detect induction or inhibition effects of tam on metabolising enzymes taking place after 7 days of chronic treatment. Due to the design of the present study, we were not able to determine whether steady-state conditions were obtained after 21 days of oral treatment.

The absence of detectable serum levels of demethylated metabolites after 3 weeks of treatment with s.c. slow release pellets was another interesting observation. To analyse this relationship in detail a separate study is needed. However, the fact that s.c. administration of tam bypasses the first pass metabolism may be of importance.

After oral administration, tam concentrations were more than twice as high in adipose tissues than in the lungs, whereas similar levels were observed in both tissues after s.c. administration.

The bioavailability of oral drugs is a multifactorial phenomenon. The rate and extent of absorption from the intestines, “release rate” from the pellet matrix, or differences in the interaction with metabolising enzymes, may explain the observed differences in the two routes of administration.

Treatment of rats with 50 mg slow release s.c. pellets produced lower concentrations of tam than the oral route, and a metabolite profile characterised by low tissue levels and undetectable serum levels of the demethylated metabolites. This is a major difference from the situation in man where the steady-state concentration of *N*-desmethyltam is higher than that of tam, though the importance of this difference remains to be elucidated [28,29].

Thus, with oral dosing, tam undergoes first-pass metabolism in the liver to active metabolites, which together may cause a stronger induction of hepatic drug-oxidising enzymes, than via the s.c. route that circumvents the first-pass effect. As a result the conversion of tam to *N*-desmethyltam may be observed primarily after oral dosing due to the induction of CYP 3A4. Moreover, it is interesting to note that many CYP 3A substrates are also P-glycoprotein (Pgp) inhibitors and this may affect the bioavailability of orally administered CYP 3A4 substrates, which include tam [30]. A thorough investigation of this complex relationship is warranted.

Conclusively, the concentrations of tam and the metabolite profile in nude rats after oral dosing were similar to those observed in other rat strains like albino Wistar and Sprague–Dawley. Moreover, this profile compares with that observed in man, whereas that in nude mice does not. The present results indicate that oral dosed nude rats may represent a preferable animal model for studying the pharmacodynamics of tam.

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