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Biochemical and pharmacological effects of toremifene metabolites

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Summary. Toremifene, a new antiestrogenic antitumor compound, has several biologically active metabolites. The hormonal effects of the main metabolites resemble those of unchanged toremifene. The main metabolite in humans, *N*-demethyltoremifene, is bound to estrogen receptors (ER), inhibits the growth of MCF-7 cells, and exerts an antiestrogenic effect similar to that of toremifene. However, its antitumor effect in vivo against dimethylbenz(a)anthracene (DMBA)-induced rat mammary cancers is weaker than that of toremifene. Didemethyltoremifene has antiestrogenic actions in mouse and rat uterus at high doses. 4-Hydroxytoremifene is bound to ER with higher affinity and inhibits MCF-7 growth at concentrations lower than those of toremifene. It has a weaker intrinsic estrogenic effect than does toremifene. The efficacy of 4-hydroxytoremifene against DMBA-induced cancers is weak except at very high doses. Oxidations of *N*-demethylated metabolites to (deamino)hydroxylated compounds and carboxylic acids are the detoxification routes of toremifene. (deaminohydroxy)Toremifene has only weak hormonal actions at high doses and carboxylated metabolites have no estrogenic/ antiestrogenic effects. The antitumor effect of toremifene in vivo is mainly due to unchanged toremifene, but hormonal effects (which may have a role in antitumor actions) are partly attributable to metabolites *N*-demethyltoremifene, didemethyltoremifene, (deaminohydroxy)toremifene, 4-hydroxy-*N*-demethyltoremifene, and 4-hydroxytoremifene, which have pharmacological properties similar to those of toremifene.

Introduction

Toremifene is a new antiestrogenic antitumor compound developed by Farmos Group Ltd. in Finland; it was developed using a rational screening system and was intended for use against breast cancer. Its basic pharmacological properties [11, 13] and clinical effects [21] have previously been described.

The pharmacokinetics of toremifene have been extensively studied in both humans and rats [1, 19]. It is excreted mainly via feces as metabolites. In humans, two main metabolites in the serum are *N*-demethyltoremifene and (deaminohydroxy)toremifene. The chemical structure of identified and proposed metabolites of toremifene and a definition of the nomenclature used are presented in Fig. 1. Most of the metabolites have been identified in the feces only.

The aim of the present study was to examine the biochemical-pharmacological activities of the identified toremifene metabolites and accordingly, to evaluate their possible role in the clinical treatment of breast cancer patients.

Materials and methods

Compounds. Toremifene and its metabolites were synthesised and purified in the Chemical Research Laboratory of Farmos Group Research Center, Oulu, Finland. The compounds were pure Z-isomers, except for carboxylic acids, which were 1 : 1 mixtures of geometric Z- and E-isomers and the pure E-isomer of toremifene. The purity all compounds was >95% and that of the main metabolites, >98%. Dimethylbenz(a)anthracene (DMBA; Sigma, St. Louis, Mo., USA) was used for the induction of mammary cancers in rats.

Cells and animals. The human estrogen receptor (ER)-positive breast-cancer cell line MCF-7 was generously provided by Dr. Charles McGrath, Michigan Cancer Foundation. Female NMRI mice and Sprague-Dawley rats were purchased from Alab (Sollentuna, Sweden) at an age of 18 days and the treatments were started on the day of their arrival. During the assays, animals were kept in the animal house on a 12-h light cycle (light from 6 a.m. to 6 p.m.) and at a temperature of 22° ± 2° C. The animals had free access to laboratory-animal food pellets (Ewos, Stockholm, Sweden) and tap water and were caged as one treatment group per cage.

Binding of new compounds to estrogen receptors. The competitive binding of [^{3}H]-estradiol and toremifene metabolites was studied according to the modified dextran-coated charcoal (DCC) method [15], with slight

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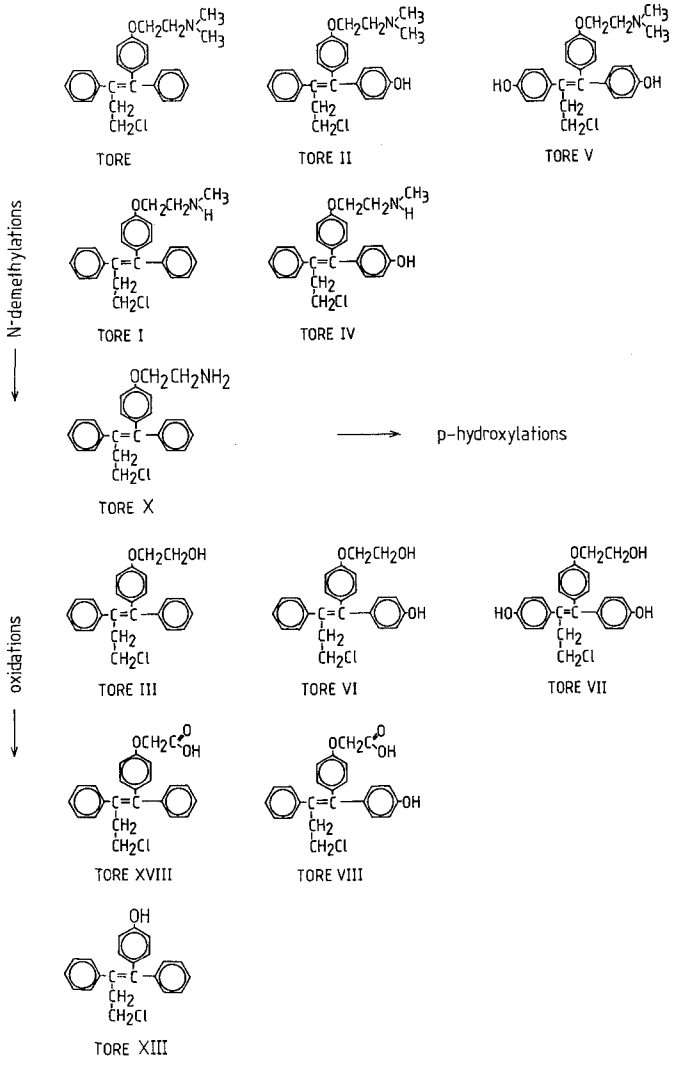

Fig. 1. The chemical structures of identified and proposed toremifene metabolites. TORE, toremifene; TORE I, *N*-demethyltoremifene; TORE II, 4-hydroxytoremifene; TORE III, (deaminohydroxy)toremifene; TORE IV, 4-hydroxy-*N*-demethyltoremifene; TORE V, 4,4′-dihydroxytoremifene; TORE VI, 4-hydroxy(deaminohydroxy)toremifene; TORE VII, 4,4′-dihydroxy(deaminohydroxy)toremifene; TORE VIII, 4-hydroxy(deaminocarboxy)toremifene; TORE X, didemethyltoremifene; TORE XIII, toremifene monophenol; TORE XVIII, (deaminocarboxy)toremifene

modifications as previously described [11]. Concentrations that inhibited 50% of estradiol binding (IC_{50}) were calculated from the log concentration vs displacement curve. The interassay coefficient of variation was 21% and that within assays, 7%.

Inhibition of MCF-7 cell growth. MCF-7 cells were cultivated in a humidified incubator at 37° C in the presence of 5% CO_2 in air. The medium was Eagle's minimum essential medium (MEM; Gibco Europe Ltd., Renfrewshire, Scotland) supplemented with nonessential amino acids (Gibco), 292 mg/l L-glutamine (Fluka AG, Buchs, Switzerland), 111 mg/l sodium pyruvate (Merck AG, Darmstadt, FRG), 0.6 µg/ml bovine insulin (Collaborative Research Inc., Lexington, Mo., USA), 25 mM HEPES buffer (Sigma, St. Louis Mo., USA), and 10 µg/ml gentamicin (Collaborative Research). Unstripped 1% fetal calf serum (Gibco) was also added to the medium. The cells were plated in 96-well dishes (Nunc, Roskilde, Denmark) in 100 µl medium. Toremifene as well as the metabolites were added to the bottoms of the wells in a small volume of ethanol; the concentration of ethanol in the cultivation medium never exceeded 0.07%.

Table 1. Binding of toremifene, tamoxifen and main metabolites of toremifene to rat uterine ER

Compound	Binding affinity (%) (estradiol = 100%)
TORE	3
TORE I	5
TORE II	64
Tamoxifen	3

Values represent the mean binding affinity of at least 5 separate determinations of IC_{50} as compared with that of estradiol

After cultivation of the MCF-7 cells for 2 or 3 days, the numbers of living cells were estimated by measurement of adenosine triphosphate (ATP) in the cells. The method indicates a good correlation between the ATP level and the number of living cells [12]. In short, the cells were disrupted by the addition of 100 µl 1% trichloric acetic acid (TCA) to the wells, which liberated ATP from the cells. An aliquot (100 µl) of the sample was transferred to buffered ATP-monitoring reagent (LKB-Wallac, Turku, Finland), which contained firefly luciferase. This changed the chemical energy of ATP to light, which was measured by a luminometer (Model 1250) equipped with a Model 2210 potentiometric recorder (both from LKB-Wallac). This method is technically easy, rapid, and sensitive and is well suited for the measurement of cell growth.

Estimation of estrogenic and antiestrogenic properties. The estrogenic and antiestrogenic properties of the toremifene metabolites were investigated by a 3-day uterotropic assay in immature mice and rats using a previously described method [8]. Toremifene and the metabolites were given s.c. to mice in sesame oil and p.o. to rats in vehicle containing 28.8 g/l polyethylene glycol 1000, 6.65 g/l, Tween-80, 1.73 g/l methyl-*p*-hydroxybenzoate, and 0.19 g/l propyl-*p*-hydroxybenzoate in distilled water. The ingredients were added to the water and the mixture was sterilized by autoclaving at 105° C for 15 min. The estrogenicity was evaluated by administration of the compounds alone at the doses indicated in Tables 3 and 4 and antiestrogenicity, by administration of the compounds together with estradiol. In this assay, estrogens increase the weight of the uterus and antiestrogens inhibit this uterotropic affect. On the 4th day the animals were anesthetised by CO_2 asphyxia and the uteri were carefully prepared, free of fat, before weighing. Animal body weights were also recorded and the relative weight of the uterus was calculated.

Estimation of antitumor activity in the DMBA-induced rat mammary-cancer tumor model. The method for investigating in vivo antitumor properties has previously been described in detail [13]. Briefly, a single p.o. dose of 12 mg DMBA suspended in sesame oil was given to 48- to 52-day-old Sprague-Dawley rats in a special isolator (Metall & Plastik GmbH, Radolfzell, FRG). After about 6 weeks the animals developed palpable tumors. When the biggest tumors were about 1 cm in diameter, treatment with the test compounds was started. The compounds were given p.o. daily for 5 weeks; tumors were individually palpated once or twice a week. The tumors were divided into growing, stable, and regressing lesions, and the number of tumors in each class was used for the estimation of effectiveness. Growing tumors showed at least 4-fold increases in size during treatment and regressing lesions decreased to <25% of their predosing size; otherwise, the tumors were considered to be stable. The numbers of new and disappearing tumors were registered during every palpation. Statistical significance was calculated using the chi-square test.

Results

The binding affinities of toremifene and its main metabolites for estrogen receptors (ER) are presented in Table 1.

Table 2. Effect of toremifene and its metabolites on the growth of MCF-7 cells in vitro

Compound	Concentration of the compounds (μM)			
	0.1	1	5	10
TORE	71.0 ± 0.8	45.6 ± 0.5	0	0
TORE I	88.4 ± 2.4	51.0 ± 2.4	0	0
TORE II	71.2 ± 3.7	68.7 ± 1.8	7.1 ± 0.9	0
TORE III	103.8 ± 1.2	80.9 ± 0	35.6 ± 0.6	4.8 ± 0.6
TORE IV	58.9 ± 6.9	46.0 ± 2.6	0.6 ± 0.9	0
TORE V	73.6 ± 0	57.1 ± 1.6	18.1 ± 5.4	0.5 ± 0
TORE VI	68.6 ± 1.5	69.6 ± 1.5	59.1 ± 6.0	11.6 ± 13.4
TORE X	108.1 ± 2.7	71.1 ± 4.0	0	0
TORE XIII	107.8 ± 7.1	101.7 ± 1.4	52.4 ± 14.2	3.0 ± 0
TOREX VIII	102.4 ± 10.7	102.4 ± 1.3	125 ± 15.4	82.5 ± 6.7
E-isomer of toremifene	88.7 ± 2.3	87.1 ± 13.7	15.3 ± 12.5	0

The numbers of living cells were measured by the ATP method [12] and are shown as a percentage of control (no hormone additions) values. Each value represents the mean ± SD of 2–4 measurements after 3 days' cultivation

Table 3. Estrogenic and antiestrogenic effects of toremifene and its metabolites in immature (20-day-old) mouse uterus in vivo

Compound	Dose of the compound		
	50 μg/kg	0.5 mg/kg	5 mg/kg
Compounds alone = estrogenicity:			
TORE	180 ± 40	303 ± 34	314 ± 34
TORE I	149 ± 59	326 ± 38	305 ± 8
TORE II	193 ± 31	335 ± 11	321 ± 35
TORE III	84 ± 8	138 ± 26	356 ± 40
TORE IV	79 ± 6	207 ± 9	261 ± 18
TORE V	66 ± 14	300 ± 4	350 ± 24
TORE VI	82 ± 32	118 ± 46	207 ± 16
TORE X	90 ± 5	370 ± 13	286 ± 46
TORE XIII	80 ± 6	98 ± 16	693 ± 61
TORE XVIII	56 ± 3	46 ± 9	52 ± 1
E-isomer of toremifene	84 ± 6	81 ± 9	327 ± 35
Compound + estradiol = antiestrogenicity:			
TORE	405 ± 65	335 ± 20	328 ± 46
TORE I	540 ± 78	392 ± 1	320 ± 27
TORE II	430 ± 49	338 ± 11	270 ± 0
TORE III	716 ± 46	769 ± 46	603 ± 36
TORE IV	519 ± 7	351 ± 78	272 ± 52
TORE V	670 ± 110	nd	383 ± 33
TOREVI	580 ± 22	569 ± 34	405 ± 85
TORE X	485 ± 25	512 ± 92	292 ± 41
TORE XIII	729 ± 39	675 ± 99	753 ± 56
TORE XVIII	597 ± 101	622 ± 68	603 ± 108
E-isomer of toremifene	680 ± 19	651 ± 161	630 ± 73

Each value represents the mean ± SD of relative uterine wet weight (mg/g body wt. × 100) obtained from 2–5 mice. Dosing route, s.c. Control values: no hormonal treatment, 70 ± 15; estradiol stimulus alone at a dose of 50 μg/kg, 730 ± 15

Table 4. Estrogenic and antiestrogenic properties of toremifene and its main metabolites in 18-day-old rat uterus

Compound	Dose of the compound			
	50 μg/kg	0.5 mg/kg	5 mg/kg	50 mg/kg
Compounds alone = estrogenicity:				
TORE	61 ± 10	111 ± 14	120 ± 14	139 ± 6
TORE I	64 ± 9	95 ± 8	121 ± 13	134 ± 13
TORE II	76 ± 5	92 ± 11	115 ± 14	134 ± 1
TORE III	71 ± 11	104 ± 6	106 ± 13	130 ± 7
E-isomer of toremifene	49 ± 5	84 ± 26	227 ± 15	204 ± 22
Compounds + estradiol = antiestrogenicity:				
TORE	167 ± 22	139 ± 24	133 ± 8	148 ± 5
TORE I	168 ± 30	135 ± 25	128 ± 9	136 ± 5
TORE II	172 ± 42	137 ± 9	127 ± 5	147 ± 14
TORE III	182 ± 49	164 ± 11	128 ± 28	139 ± 10
E-isomer of toremifene	221 ± 16	185 ± 39	193 ± 29	216 ± 63

Each value represents the mean ± SD of relative uterine wet weight (mg/g body wt. × 100) obtained from 5–10 animals. Control values: no hormonal treatment, 69 ± 14; estradiol stimulus alone at a dose of 50 μg/kg s.c., 203 ± 48

Table 5. Antitumor effect of toremifene and its main metabolites in DMBA-induced rat mammary cancer

Group	Growing tumors (*n*)	Stable tumors (*n*)	Regressing tumors (*n*)	New tumors (*n*)	*P* ($\times^2$ test)
Control	3.8 ± 2.7	1.3 ± 1.4	0.1 ± 0.4	3.2 ± 2.1	
TORE 3 mg/kg	1.3 ± 1.3	2.5 ± 2.4	1.0 ± 0.5	0.6 ± 1.3	<0.05
TORE I 3 mg/kg	3.8 ± 2.1	1.4 ± 1.5	0.2 ± 0.6	2.9 ± 2.8	NS
TORE II 3 mg/kg	2.0 ± 2.8	3.2 ± 1.9	0.2 ± 0.5	2.4 ± 3.4	NS
15 mg/kg	2.6 ± 3.1	1.8 ± 1.3	1.6 ± 1.1	1.8 ± 1.5	NS
E-isomer of toremifene	2.0 ± 1.4	1.5 ± 1.3	0.5 ± 1.0	0.3 ± 2.1	NS

Treatment was given daily for 5 weeks p.o. as described in Materials and methods. Each value represents the mean ± SD of at least 5 rats and at least 30 tumors and expresses the number of tumors/animal. Statistical significance is shown in comparison with control values. NS, not significant

The effect of toremifene and its metabolites on MCF-7 cell growth in vitro is shown in Table 2.

Estrogenic and antiestrogenic properties of toremifene and its metabolites in the mouse uterus are listed in Table 3. In the mouse uterus all synthesised metabolites were screened in a small number of animals. The main metabolites were also subjected to rat uterotropic assay using a larger number of animals. The results of the rat assays are presented in Table 4. The effectiveness of toremifene and its main metabolites were studied in DMBA-induced rat mammary tumors. The numbers of growing, stable, and regressing tumors in different treatment groups after 5 weeks' daily administration are shown in Table 5.

Discussion

Different metabolic steps influence the biochemical properties of toremifene in a variable manner. *N*-demethylation has no significant effect on the ER-binding properties, but 4-hydroxylation markedly increases the binding affinity (Table 1). This is accordance with previous findings obtained using related tamoxifen metabolites [2, 4, 17]. Further oxidative metabolic steps of the nitrogen side chain that yield (deaminohydroxy) derivatives decrease this binding, indicating the importance of side-chain nitrogen in binding to ER (Table 1). A very similar influence of metabolic reactions on the binding affinities of tamoxifen metabolites has previously been shown [6, 22].

The antitumor activity of toremifene and its metabolites against MCF-7 cells in vitro did not correlate well with ER-binding properties; e.g., 4-hydroxytoremifene was no more active than toremifene in MCF-7 cells at similar concentrations (Table 2). This finding is different from those obtained for tamoxifen metabolites, which show a good correlation between receptor-binding and MCF-7 growth-inhibition activities [3, 16]. The carboxylic acid metabolites had no activity in MCF-7 cells (Table 2). Toremifene and its metabolites (analogous to tamoxifen) have species-specific hormonal actions: they are more estrogenic in mice than in rats (Tables 3, 4).

N-Demethyltoremifene closely resembles the parent compound in its hormonal properties. 4-Hydroxylation, in analogy to tamoxifen, gives antiestrogens with low intrinsic estrogenic activity at low and moderate doses (Tables 3, 4) [10]. At very high doses of about 50 mg/kg, the estrogenicity tends to increase. Especially interesting as antiestrogens are 4-hydroxytoremifene and 4-hydroxy-*N*-demethyltoremifene. *p*-Hydroxylation of 4-hydroxytoremifene to 4,4′-dihydroxytoremifene yields weak estrogen with almost no antiestrogenic effect (Table 3). Similar properties for dihydroxylated tamoxifen have been reported [8].

Didemethyltoremifene is an antiestrogen in mice at a dose of 5 mg/kg (Table 3). This compound is detectable in human serum under steady-state conditions (unpublished results) and it can – in small part – be responsible for the antiestrogenicity of toremifene in vivo. Side-chain hydroxylation of toremifene yields compounds [alcohols = (deaminohydroxy)-derivatives] that have weak hormonal activities at high doses. (deaminohydroxy)Toremifene shows an interesting species specificity: it is purely estrogenic in the mouse uterus but has definitive antiestrogenic activity in the rat uterus, comparable with that of toremifene at doses of 5 and 50 mg/kg.

No data are available on the hormonal effects of this compound in humans. As this compound is one of the main metabolites in human serum, this result is very interesting. However, due to the low hormonal and antitumor activity of toremifene at low doses, oxidation of the nitrogen-containing side chain can be considered to be the detoxification pathway. Estrogenic properties for the analogous tamoxifen metabolite (metabolite Y) have been reported [14]. Further metabolism of alcohols to carboxylic acids yields compounds with no estrogen or antiestrogen-like properties. Carboxylic acid metabolites of tamoxifen have not been described, although they are almost certainly formed during administration of the compound in humans and in animals [19].

The side chain of toremifene may also be metabolically cleaved to yield toremifene monophenol. The concentrations of this metabolite are very low in rats and not detectable in human samples [1, 19]. An analogous metabolite has also been found for tamoxifen (metabolite E), and it has been shown to be purely estrogenic [6], as was toremifene monophenol in the present study.

The antitumor effect of toremifene in DMBA-induced rat mammary cancer is well documented [13, 18]. The main metabolites of toremifene exert weaker antitumor activity than does the parent compound on DMBA-induced rat mammary cancers. It is interesting, however, that 4-hydroxytoremifene markedly increased the number of regressing tumors at the high dose of 15 mg/kg, although it could not suppress the appearance of new tumors and had almost no effect at lower doses. This finding is in agreement with the observations of Jordan and Allen [7]: the rapid clearance of 4-hydroxytamoxifen results in lower antitumor activity than could be expected on the basis of its strong antiestrogenic effect in the uterotropic test. One can also speculate that 4-hydroxylated metabolites undergo first-pass metabolism in the liver and then appear in the circulation and can penetrate to the nuclear compartment of cancer cells only after high doses. In contrast to these results, monohydroxytamoxifen has been reported to have higher activity against the GR hormone-dependent mouse mammary tumor than does tamoxifen [20]. In the latter study, animals simultaneously received estrone and progesterone, which may explain the discrepancy.

4-Hydroxy-*N*-demethyltoremifene is formed when both *p*-hydroxylation and *N*-demethylation have occurred. This metabolite has not been found in human samples, but it is strongly antiestrogenic, as can be expected from its chemical structure.

Many substituted antiestrogenic triphenylethylenes have two geometric isomers. Toremifene is a pure Z-isomer (= *trans*). The E-isomer (= *cis*) was also studied in the present work. It was less active against MCF-7 cells than was toremifene and was purely estrogenic in both mouse and rat uterus. However, this compound, inhibited the appearance of new tumors in DMBA-induced mammary cancer, although it was otherwise inactive. The E-isomer of tamoxifen also lacks antiestrogenic properties [5]. Jordan et al. [9] have reported differences between geometric isomers of several antiestrogens and confirmed the negligible antiestrogenic properties of the E-isomer of tamoxifen. Our results show that E-isomers of tamoxifen and toremifene have similar pharmacological properties.

Most of the metabolites characterized in the present study have been identified in feces or are hypothetical metabolites. *N*-Demethyltoremifene and (deaminohydroxy)toremifene are the main metabolites in human serum [1]. Analogous to tamoxifen and its main metabolite [6], the concentrations of *N*-demethyltoremifene are about 2 times those of toremifene, 1.6 and 0.8 μg/ml, respectively, after a daily dose of 60 mg under steady-state con-

ditions. Therefore, *N*-demethyltoremifene may be partly responsible for the antiestrogenic (but not the antitumor) effects of toremifene in humans.

According to very preliminary (unpublished) results, the concentrations of *N*-demethyltoremifene are remarkably low in tumor tissue; poor tumor distribution could therefore explain the weak antitumor action of this metabolite. (Deamino)hydroxytoremifene concentrations in human serum are about 10% of those of the parent compound under steady-state conditions. Taking into account the low, albeit interesting, hormonal activity of this metabolite at low doses, it might not be responsible for either the hormonal or the antitumor activity noted during toremifene treatment. Anttila et al. [1] did not find 4-hydroxytoremifene in human serum, even after high toremifene doses. However, this theoretically interesting metabolite was measurable in tumor tissue (unpublished results) and may therefore have some biological activity during high-dose toremifene treatment. At high doses, didemethyltoremifene is almost as strongly antiestrogenic as toremifene, but only low concentrations of this metabolite have been found in human serum samples. The importance of this metabolite in toremifene therapy might therefore be low.

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