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ORIGINAL ARTICLE

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A novel therapeutic trial of homogentisic aciduria in a murine model of alkaptonuria

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Abstract Alkaptonuria is a rare autosomal recessive disorder characterized by homogentisic aciduria, ochronosis, and arthritis. Although a deficiency of homogentisic acid 1,2-dioxygenase has recently been confirmed at the molecular level, no effective treatment regimen has yet been developed for this disorder. In the present study, 2(-2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC), a potent inhibitor of p-hydroxyphenylpyruvate dioxygenase (which catalyzes the formation of homogentisic acid from p-hydroxyphenylpyruvic acid) was adopted as a possible therapeutic agent for alkaptonuria. NTBC dose-dependently reduced the urinary output of homogentisic acid in a murine model of alkaptonuria that had been created with ethylnitrosourea. These findings suggest that NTBC may be the first potent pharmacotherapeutic agent for alkaptonuria.

Key words Alkaptonuria · Homogentisic acid · Treatment · NTBC · Murine model · Pharmacokinetics · Inborn error of metabolism

Introduction

Alkaptonuria (MIM no. 203500) represents a classical and widely known example of inborn errors of metabolism first proposed by Garrod (1908) at the beginning of the twentieth century. It is a rare autosomal recessive disorder characterized by homogentisic aciduria (which causes a darkening of urine on prolonged exposure to air), ochronosis, and arthritis (La Du 1995). The disease is caused by a deficiency of homogentisic acid 1,2-dioxygenase (HGO. EC 1.13.11.5), which converts homogentisic acid (HGA) to maleylacetoacetic acid in the tyrosine degradation pathway (La Du et al. 1958) (Fig. 1). In 1996, Fernandez-Cañón and colleagues succeeded in verifying this phenotype at the molecular level.

Although alkaptonuria has been considered a relatively benign disorder, ochronotic arthritis can often be devastating and may result in patients becoming completely bedridden later in life (Kihara et al. 1994, La Du 1995, Fernandez-Cañón et al. 1996). No effective treatment for alkaptonuria has yet been found, despite various attempts to find a remedy (La Du 1995). In this regard, we have recently noted that 2 (-2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC), a potent inhibitor of p-hydroxyphenylpyruvate dioxygenase (HPPD. EC 1. 13. 11. 27), which catalyzes the formation of HGA from p-hydroxyphenylpyruvic acid (Lindstedt et al. 1992) (Fig. 1), may be a suitable therapeutic agent for alkaptonuria. NTBC was used in five patients with hereditary tyrosinemia type 1 (fumarylacetoacetate hydrolase deficiency) (Lindstedt et al. 1992), and its efficacy, and safety were also demonstrated in a murine model of the same disorder (Grompe et al. 1995).

Here we describe the oral use of NTBC in a murine model of alkaptonuria (created with ethylnitrosourea by Montagutelli et al. 1994) and demonstrate that NTBC clearly corrects the underlying metabolic defect. Our findings suggest that NTBC may therefore be the first effective pharmacotherapeutic agent for alkaptonuria.

Materials and methods

Animals

Alkaptonuric mice were kindly provided by Dr. X. Montagutelli, of the Pasteur Institute. Mutant mice were

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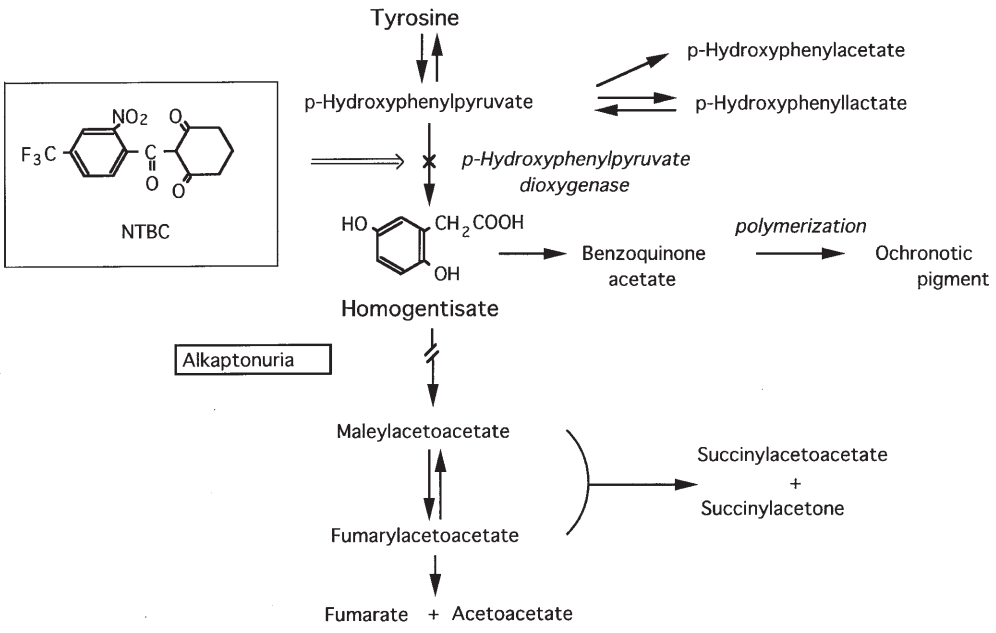

Fig. 1 The action site of 2(-2-nitro-4-trifluoromethylbenzoyl)-1,3 cyclohexanedione (*NTBC*) in tyrosine catabolism, including the steps required for the formation of ochronotic pigment in alkaptonuria. *Break in arrow*, block in alkaptonuria

identified by the discoloration seen when a droplet of urine was deposited on filter paper impregnated with 0.5 M NaOH and dried prior to use (Montagutelli et al. 1994). They were housed in metallic cages at 23°C under specific pathogen-free conditions, fed on CE-2 chow (Clea Japan, Tokyo), and used when they were 5–6 months of age.

Reagents

NTBC was synthesized in the laboratories of Sumitomo Pharmaceuticals (Osaka, Japan) for our experimental use. HGA and p-hydroxyphenyllactic acid (PHPL) were purchased from Sigma Chemical (St. Louis, MO, USA). p-Hydroxyphenylpyruvic acid (PHPP) and p-hydroxyphenylacetic acid (PHPA) were obtained from Wako Pure Chemical Industries (Osaka, Japan) and Nacalai Tesque (Kyoto, Japan), respectively. n-Heptadecanoic acid was purchased from Pierce Chemical (Rockford, IL, USA). Bis (trimethylsilyl) trifluoroacetamide (BSTFA) and hydroxylamine HCl were obtained from Pierce Chemical and Wako Pure Chemical Industries, respectively.

NTBC treatment

NTBC was dissolved in 50 mM $NaHCO_3$ and kept at −20°C until use, as previously reported (Grompe et al. 1995). The mice received various doses of NTBC administered orally by a stomach tube either once or repeatedly.

Determination of NTBC in plasma

Fifty microliters of plasma was pipetted into plastic vials containing 50 µl of 50 mM $NaHCO_3$, and 50 µl of acetonitrile was added for protein precipitation. The vials were then vortexed and centrifuged for 5 min at 15,000 rpm at 4°C. An aliquot (50 µl) of the supernatant was injected into a high-performance liquid chromatograph (TOSOH SC-8010, Tosoh, Tokyo, Japan) with an ultraviolet (UV) detector and a Sumipax ODS A-212 column (0.6 ×15 cm; Sumika Chemical Analysis Service, Osaka, Japan). The mobile phase consisted of an aqueous solution of 10 mM sodium phosphate buffer (pH 7.40)/methanol (60:40, v/v). The flow rate was set at 1.0 ml/min. The peak NTBC was retained for 11.0 min. The peak area of NTBC was linearly proportional to the amount of NTBC added to plasma at a range of 0–200 µg/ml. The minimum amount measured was 0.1 µg NTBC / ml plasma. All plasma samples and solutions for calibration were prepared in duplicate.

Analysis of urinary organic acids

Urine was collected from the mice in the metabolic cages for 12 or 24 h in the presence of sodium metabisulfite (at least 1 mg ml^{-1}) and liquid paraffin, and was then kept at −20°C until analysis. An aliquot of urine was added, in duplicate, to n-heptadecanoic acid, an internal standard, and was then extracted with ethylacetate and diethyl ether. The combined extracts were concentrated to dryness under nitrogen gas, and trimethylsilylated with BSTFA for 1 h at 60°C before being submitted to gas chromatography/mass spectrometry (GC/MS). For the analysis of PHPP, the urine was reacted with hydroxylamine HCl prior to trimethylsilylation.

The GC/MS analysis was performed on a Hitachi G-3000/M-2000A/ M-0201 apparatus (Hitachi, Tokyo, Japan). The chromatographic separation was carried out on a DB-17 capillary column (15 m × 0.32 mm i.d., 0.5 µm film thickness. J and W Scientific, Folsom, CA, USA), using helium as the carrier gas. The temperature was programmed from 100°C to 260°C at 6°C /min. The initial temperature was maintained for 4 min. The temperature range for the HPPP

analysis was 150°–260°C. The mass spectrometer was operated in the electron impact mode at an ion source temperature of 150°C and at an electron voltage of 70 eV. Four organic acids were assayed by selected ion monitoring. The monitor ions for PHPA, PHPL, HGA, and PHPP were m/z 296, 308, 384, and 396, respectively.

We prepared the standard curves for the four organic acids in duplicate each time. The quantitative values for the organic acids were calculated by determining the peak area relative to the internal standard. The ratio was found to be linearly proportional to the amount of each organic acid added to distilled water in the range used for the analysis.

Other biochemical analyses

To examine the blood chemistry of the mice (bilirubin, aspartate aminotransferase [AST], creatinine, and amino acids), blood was collected from the orbital venous plexus with a capillary tube with the animals under general anaesthesia, and was immediately mixed with ethylenediaminetetraacetate (EDTA)-2Na. The plasma was frozen at −20°C until used for analysis. Amino acid analysis of the plasma was done on a Hitachi automated amino acid analyzer.

Histology

The animals were anesthetized with diethyl ether and bled to death, and the liver, kidney, lumbar spine, knee, and ear were removed. Histology samples were fixed in 10% neutral buffered formalin, and stained with hematoxylin-eosin.

Results

Kinetics of a single oral dose of NTBC in control mice

One hundred μg of NTBC was given orally to 15 control mice, and the plasma concentration was observed 1, 3, 5, 8, and 12 h after this oral administration (Fig. 2). The plasma NTBC concentration reached a maximum at 1 h and gradually decreased thereafter. Trace amounts of NTBC remained in the blood 12 h after administration.

Metabolic effects of a single oral dose of NTBC in alkaptonuric mice

The urinary excretion of HGA, the hallmark metabolite of alkaptonuria, from alkaptonuric mice was markedly elevated during the control period (i.e., 24 h before dosing with NTBC began) (Fig. 3). The precursor intermediates of HGA (i.e., PHPP, PHPL, and PHPA) were also elevated at this time, compared with levels in the control mice. After a single oral 100-μg dose of NTBC, a significant reduction in urinary HGA excretion and, inversely, a significant elevation of the three precursor intermediates were observed within 24 h.

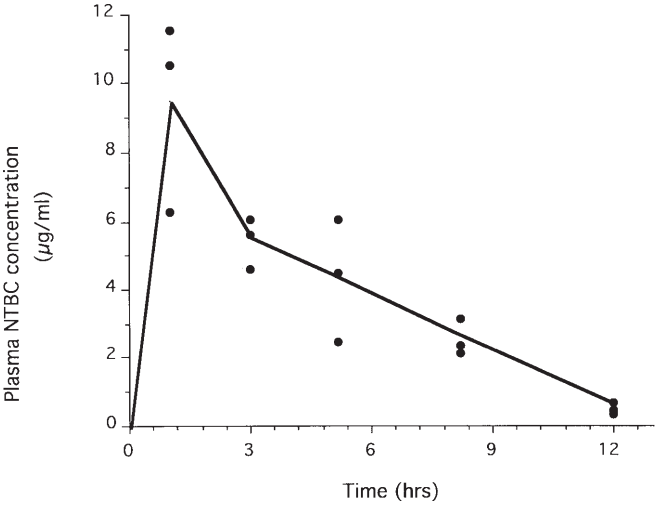

Fig. 2 Kinetics of a single 100-μg oral dose of NTBC in control mice. NTBC was well absorbed, and the maximum concentration in plasma was reached 1 h after dosage. Trace amounts of NTBC remained in the blood 12 h after administration

A single 100-μg dose of NTBC to an alkaptonuric mouse caused a dose-dependent reduction in urinary HGA output (Fig. 4). A single 100-μg dose of NTBC reduced the urinary HGA output to less than 2% of the pretreatment level 12–24 h after dosing. With any dose of NTBC, the maximum reduction in urinary HGA output was observed 12–24 h after dosing.

In the time course of the urinary excretion of four tyrosine catabolites (i.e., PHPP, PHPL, PHPA and HGA), the peak PHPP value was reached within 12 h after a single oral 25-μg dose of NTBC, while the peak values for PHPL and PHPA were reached later, at 12–24 h after dosing (Fig. 5).

Chronic effects of NTBC on biochemical and histologic findings in alkaptonuric mice

With the repeated administration of NTBC 25 μg once daily (except at weekends) for 4 weeks in four alkaptonuric mice, there was a continuous reduction in urinary HGA output (Fig. 6).

Table 1 shows the effects of 5-week NTBC administration on plasma tyrosine levels and histological findings in the liver and kidney in alkaptonuric and control mice. Plasma tyrosine levels increased by about three times compared with levels before treatment in the NTBC-treated control mice, and by four to five times in alkaptonuric mice. Histologically, mild non-specific changes were observed in three of six livers and in one of six kidneys examined. No ochronotic joint lesions were observed in any of the alkaptonuric mice. X-ray examination of three alkaptonuric mice at age 13 months showed no calcification of the cartilage and no osteoarthritic changes in the knee and spine.

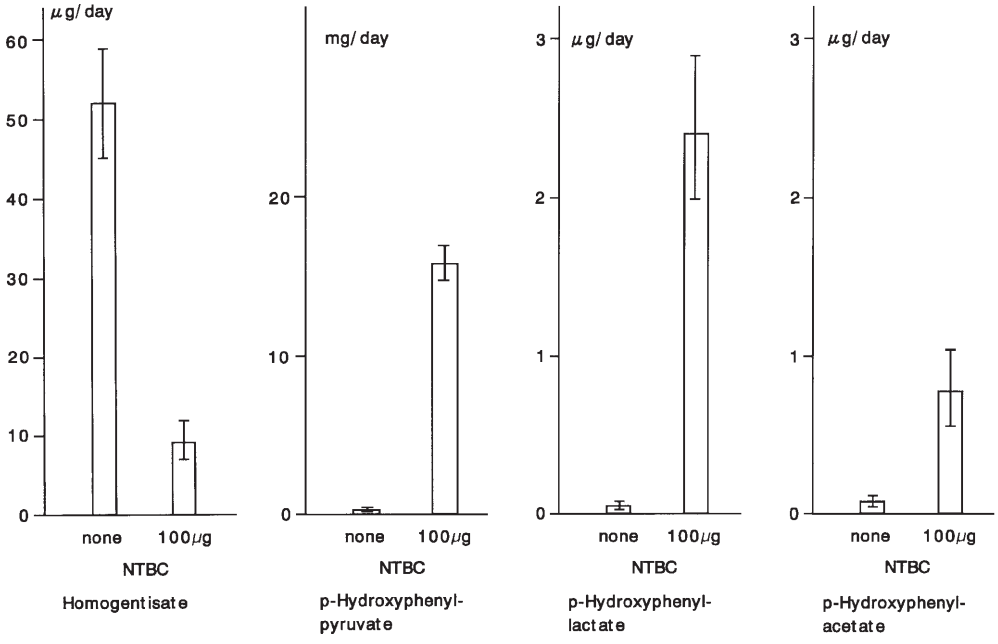

Fig. 3 Urinary excretion of homogentisic acid (HGA), as homogentisate, and three precursor intermediates of HGA in alkaptonuric mice 24 h before and 24 h after single oral 100-μg administration of NTBC. Values are expressed as means ± SEM

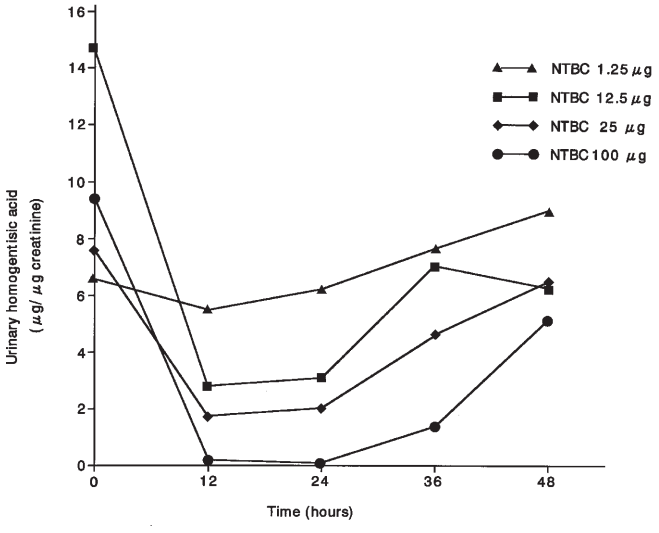

Fig. 4 Effects of various single oral doses of NTBC *triangles*, 1.25 μg; *squares*, 12.5 μg; *diamonds*, 25 μg; *dots*, 100 μg) on urinary HGA excretion in an alkaptonuric mouse

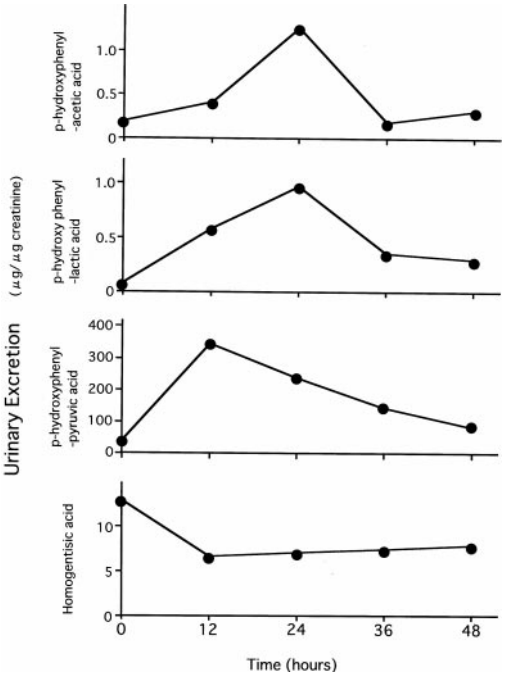

Fig. 5 Time course of the urinary excretion of HGA and its precursor intermediates in an alkaptonuric mouse after a single oral 25μg dose of NTBC. Peak excretions of p-hydroxyphenyllactic acid and p-hydroxyphenylacetic acid occurred later than that of p-hydroxyphenylpyruvic acid

Discussion

Recent in-vitro studies of rat liver HPPD have shown that NTBC is a reversible inhibitor of this enzyme (Ellis et al. 1995, 1996). In the present study we confirmed this effect in vivo, showing an elevation of urinary PHPP, PHPL, and PHPA output and plasma tyrosine concentration after NTBC administration in control mice. Such an NTBC-induced metabolic profile is reminiscent of the HPPD-deficient mouse strain described by Endo et al. (1991). Furthermore, we also showed a distinct reduction in urinary HGA output after NTBC administration to alkaptonuric mice. NTBC is thus the first compound that appears to have a definite pharmacotherapeutic effect on homogentisic aciduria in alkaptonuria.

However, the effectiveness of NTBC in treating ochronosis remains unknown, because no ochronotic lesions were observed in the alkaptonuric mice, as previously reported (Kamoun et al. 1992). The lack of ochronosis in alkaptonuric mice may reflect a species-specific characteris-

Table 1 Effects of 5-week administration of NTBC on biological parameters in plasma and histological findings in alkaptonuric and control mice

	Alkaptonuric mice		Control mice	
	NTBC (+) (n = 4)	NTBC (−) (n = 6)	NTBC (+) (n = 2)	NTBC (−) (n = 2)
Plasma[a]				
Tyrosine (nmol/ml)	414.9 ± 61.0	91.6 ± 11.8	257.2, 327.2	92.6, 103.0
AST (U/I)	69.5 ± 8.4	64.0 ± 8.6	90	123, 66
Creatinine (mg/dl)	0.3 ± 0	0.4 ± 0.1	0.3	0.3, 0.3
Histology				
Liver				
No abnormality	2	1	1	2
Focal necrosis	2	3	1	0
Lymphocytic infiltration	0	2	0	0
Kidney				
No abnormality	4	5	2	2
Severe glomerulonephritis	0	1	0	0

NTBC, 2(-2-Nitro-4-trifluoromethylbenzoyl)-1,3 cyclohexanedione; AST, aspartate aminotransferase
[a] Values are expressed as means ± SEM

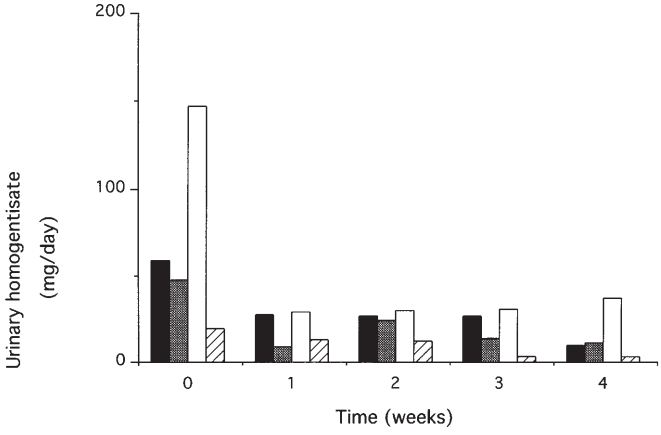

Fig. 6 Effect of 4-week administration of NTBC (25 μg/day, except weekends) on urinary HGA excretion, as homogentisate, in four alkaptonuric mice. *Black bars*, alkaptonuric mouse #1; *gray bars*, #2; *white bars*, #3; *hatched bars*, #4

tic (i.e., mice can endogenously synthesize ascorbic acid; Levine 1986). Ascorbic acid impedes the conversion of HGA to benzoquinoneacetate, an intermediary metabolite in oxidation (Fig. 1), and inhibits the generation of oxygen radicals in the development of ochronotic arthritis (Martin and Batkof 1987). The effect of ascorbic acid on the amount of HGA excreted by patients with alkaptonuria was inconclusive (Sealock et al. 1940; Neuberger et al. 1947, Wolff et al. 1989; Simoni et al. 1994; Pradeep et al. 1996). We presume that NTBC administered to alkaptonuric patients has a beneficial effect on ochronosis as well, by blocking the biosynthesis of HGA, the source of ochronotic pigments.

Attempts to treat alkaptonuria with NTBC could be aimed at either treating the disease or preventing its complications. Accordingly, NTBC could be used to relieve the intolerable arthralgia in patients with alkaptonuria, based on the assumption that it may suppress the oxygen radical-induced inflammation in connective tissues. NTBC administered over the long term during asymptomatic periods may also delay or prevent complications, by suppressing the progression of pathologic changes in connective tissue.

Grompe et al. (1995) observed no side effects, no teratogenicity, and no tumorigenicity in mice with hereditary tyrosinemia type I treated with NTBC. Our histological findings support a part of their observations. Furthermore, no side effects were encountered in patients with hereditary tyrosinemia type I who were successfully treated with an oral daily dose of NTBC of 0.1–0.6 mg/kg (10%–60% of the dose used for mice in the present study) (Lindstedt et al. 1992). NTBC-induced hypertyrosinemia may have no adverse effects, as HPPD deficiency can be compatible with normal development in some humans (Mitchell et al. 1995), and, further, HPPD-deficient mice were also apparently healthy (Endo et al. 1991).

For either curative or preventive purposes, the use of NTBC together with ascorbic acid would be preferable, since, as pointed out by Sealock et al. (1940), it is possible that adequate tissue concentrations of ascorbic acid may prevent the deposition of ochronotic pigment.

These findings suggest that NTBC may be a promising agent for the treatment of alkaptonuria. Clinical trials of NTBC would be warranted in the future.

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