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ORIGINAL ARTICLE

In vitro assessment of cytochrome P450 inhibition and induction potential of tanespimycin and its major metabolite, 17-amino-17-demethoxygeldanamycin

Jinping Gan · Peggy Liu-Kreyche · W. Griffith Humphreys

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Abstract
Purpose To assess the inhibition and induction potential of tanespimycin and its major metabolite, 17-amino-17-demethoxygeldanamycin (17-AG) on cytochrome P450 (CYP) enzymes.
Methods The inhibitory effect of tanespimycin and 17-AG on various CYP enzymes was determined in human liver microsomes. The inductive effects of tanespimycin and 17-AG on CYP1A2, CYP2B6, and CYP3A4/5 were determined in cultured primary human hepatocytes.
Results Tanespimycin did not inhibit the activities of CYP1A2, 2A6, 2B6, and 2E1 up to a concentration of 60 μM, while it moderately inhibited CYP3A4/5 and 2C19, and weakly inhibited CYP2C8, 2C9, and 2D6. In addition, its inhibition on CYP3A4/5 was time-dependent. 17-AG moderately inhibited the activities of CYP3A4/5 and CYP2C19, but did not inhibit other CYPs up to a concentration of 30 μM. The inhibition of CYP3A4/5 by 17-AG was not time-dependent. Tanespimycin and 17-AG did not significantly induce the activities of CYP1A2, CYP2B6, or CYP3A4/5 in cultured human hepatocytes at concentrations up to 40 and 20 μM for tanespimycin and 17-AG, respectively.
Conclusions Tanespimycin together with its active metabolite, 17-AG are moderate inhibitors of CYP3A4/5 and CYP2C19, but not inducers of CYPs. Therefore, co-administration of tanespimycin has the potential to increase the exposure of substrates of CYP2C19 and CYP3A4/5.

J. Gan (✉) · P. Liu-Kreyche · W. G. Humphreys
Pharmaceutical Candidate Optimization,
Bristol-Myers Squibb Research and Development,
Princeton, NJ 08543, USA
e-mail: jinping.gan@bms.com

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Introduction

Heat shock protein 90 (Hsp90), together with other co-chaperone proteins (e.g., Hsp70, Hip, Hop, Hsp40, Cdc37, p23), assists the folding, maturation, stability, and trafficking of many client proteins involved in oncogenesis, including steroid hormone receptors, signaling kinases, transcription factors, and telomerase [1, 2]. It is proposed that the inhibition of Hsp90 would disrupt oncogenic signaling pathways and sensitize resistant tumors, thus Hsp90 inhibitors are attractive combination partners with chemotherapeutics agents including cytotoxics and targeted agents. Tanespimycin (17-AAG), a derivative of the natural product geldanamycin, inhibits Hsp90 by binding to its ATP binding pocket, thus disrupting its binding with its co-chaperone Hsp70 [1]. Tanespimycin is currently under clinical investigation against multiple tumor types.

Tanespimycin undergoes CYP3A4/5-mediated metabolism primarily on the allylic side chain to form an epoxide, a diol, and a de-alkylated product, 17-AG [2, 3]. In vitro assays demonstrated that 17-AG is as active as tanespimycin in Hsp90 inhibition [4]. The structures of tanespimycin and its major metabolites are shown in Fig. 1.

Modulations of CYP activities, either inhibition or induction by tanespimycin, may result in potential increase or decrease of the systemic exposures of chemotherapeutic combination partners or other coadministered drugs resulting in decreased efficacy or increased toxicity. Because many chemotherapeutic agents have narrow therapeutic ranges, the assessment of the inhibition and induction

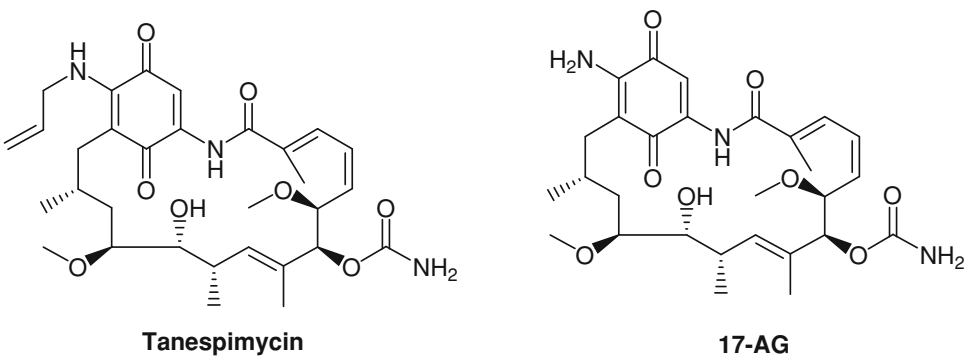

Fig. 1 Chemical structures of tanespimycin and 17-AG

potential of CYP activities by tanespimycin and its active metabolite, 17-AG, becomes important.

The objective of this study was to evaluate the metabolic drug–drug interaction potential of tanespimycin through investigation of tanespimycin and 17-AG as a perpetrator for drug–drug interaction via evaluation of P450 enzyme inhibition or induction potential in human liver microsomes (HLM) and primary human hepatocytes.

Materials and methods

Tanespimycin (17-allylamino-17-demethoxygeldanamycin, KOS-953) was manufactured at Ash Stevens™ (Riverview, MI). Pooled HLM from 15 donors were obtained from CellzDirect (Austin, TX). P450 enzyme-specific marker substrates, metabolites, and positive control inhibitors were purchased from Sigma–Aldrich (St Louis, MO), BD Gentest (Woburn, MA), Toronto Research Chemicals (North York, Ontario, Canada), and Cerillant (Round Rock, TX). All other chemicals and solvents were at the highest chemical grade available. The stock solutions of tanespimycin (15 mM) were prepared in 90% methanol: 10% DMSO (dimethyl sulfoxide) (v:v) and stored at approximately 4°C before usage.

For the induction studies, phosphate buffered saline and Geltrex® were purchased from Invitrogen (Gibco, Grand Island, NY). Insulin transferrin media supplement (ITS+) and ECM proteins (collagen Type 1 and matrigel) were purchased from BD Biosciences (San Jose, CA). Tissue culture media (Dulbecco's Modified Eagle's Media (DMEM) and Chee's), dexamethasone (DEX), Hanks' Balanced Salt Solution (HBSS), DMSO, 3-methylcholanthrene (3-MC), phenobarbital (PB), and rifampicin (RIF) were procured from Sigma Chemical Co. (St. Louis, MO).

Assessment of potential of tanespimycin to inhibit P450 enzymes

To assess the potential of tanespimycin to inhibit the major P450 enzymes, probe substrates specific for each enzyme were used to assay the activity of each P450 enzyme in HLM in the presence and absence of tanespimycin. The concentrations of P450 probe substrates (selective enzyme probed) were 50 μM for phenacetin (CYP1A2), 1 μM for coumarin (CYP2A6), 125 μM for bupropion (CYP2B6), 5 μM for paclitaxel (CYP2C8), 140 μM for tolbutamide (CYP2C9), 50 μM for (S)-mephenytoin (CYP2C19), 5 μM for dextromethorphan (CYP2D6), 50 μM for chlorzoxazone (CYP2E1), 5 μM for midazolam (CYP3A4/5, $CYP3A_M$), and 50 μM for testosterone (CYP3A4/5, $CYP3A_T$).

The assay procedures are similar to what was recently reported in the literature [5, 6]. Incubations were performed at approximately 37°C in with a total incubation volume of 0.5 mL. After pre-equilibration of HLM in 100 mM potassium phosphate buffer (pH 7.4), six concentrations of test inhibitor and probe substrates were added and reactions were initiated by addition of cofactor (1 mM NADPH). In addition, potential time-dependent inhibition of CYP enzyme activities by tanespimycin was determined as described above with the exception of a 30-min preincubation with test inhibitor and NADPH prior to reaction initiation with probe substrate and additional NADPH. All reactions were terminated by the addition of the appropriate extraction reagent(s), vortexed, and centrifuged at approximately 3,000 revolutions per minute (rpm) for 5–10 min to sediment the precipitated protein. The supernatants were transferred to new deep well plates, evaporated to dryness under a stream of nitrogen gas, reconstituted in 75 or 100 μL of appropriate reconstitution solution, and vortexed briefly before submitting for analysis.

Two LC–MS/MS systems were used for quantification of metabolites of the probe substrates. For analyte separations, Shimadzu HPLC system (Shimadzu Scientific Instruments, Columbia, MD) equipped with a CTC-PAL autosampler (LEAP Technologies, Carrboro, NC) was used. A Thermo TSQ Quantum mass spectrometer (Fremont, CA) was used for the CYP1A2, 2C9, 2C19, 2D6, and 3A assays. An Applied Biosystems 4000Q-trap mass spectrometer (Ontario, Canada) was used for CYP2A6, 2B6, and 2C8 assays. The individual P450-specific metabolites

were monitored by multiple reaction monitoring (MRM) as described in the literature [5, 6]. The concentration of P450-specific metabolites in each sample was quantified using the appropriate calibration curve and stable isotope-labeled metabolites as internal standards.

For ensure assay quality, incubations with the addition of appropriate positive control inhibitors (both competitive and time-dependent) were performed at single inhibitor concentrations at about the respective IC50 values.

Assessment of potential of tanespimycin to induce P450 enzymes

The potential of tanespimycin and 17-AG to induce P450 enzymes was investigated by assessing the enzyme activity and mRNA levels of CYP1A2, 2B6, and 3A in primary cultures of human hepatocytes after a 3-day treatment with tanespimycin or 17-AG. Primary cultures of human hepatocytes were prepared from human liver tissue from three donors. Human hepatocytes were isolated as described previously LeCluyse, et al. [7]. Following isolation, hepatocytes were resuspended in DMEM–Ham's F12 containing 5% fetal calf serum, insulin (4 μg/mL), and DEX (1 μM), added to 60-mm NUNC Permanox® dishes (~4×10^6/dish) or T-75 flasks (~12×10^6/flask) coated with a simple collagen, type I, substratum, and allowed to attach for 3 to 6 h at 37°C in a humidified chamber with 95%/5% : air/CO_2. After attachment, culture vessels were swirled and medium containing debris and unattached cells was aspirated. Fresh, ice cold, serum-free William's E. Medium containing 50 nM DEX, 6.25 μg/mL insulin, 6.25 μg/mL transferrin, 6.25 ng/mL selenium (ITS+), and 0.35 mg/mL Geltrex® were added to culture vessels, which were immediately returned to a humidified chamber. Media was changed on a daily basis thereafter. Cultures of hepatocytes were maintained for 36–48 h prior to treatment with tanespimycin, 17-AG, vehicle, and positive controls.

Human hepatocyte cultures were treated for three consecutive days with media containing tanespimycin (2, 20, 30, and 40 μM), 17-AG (1, 3, 10, and 20 μM), solvent controls, or prototypical inducers. 3-MC (2 μM), PB (1,000 μM), and RIF (10 μM) were the prototypical inducers used as positive controls for the human hepatocyte cultures. Negative control cultures were treated with vehicle (0.1% DMSO).

Approximately 24 h after the final treatment, microsomes and cell lysates were prepared from each culture, based on the methods described by Wortelboer et al. [8]. The enzyme activity in microsomal samples was determined by incubating microsomal samples with probe substrates for 10 min at 37°C in a final volume of 0.4 mL. The enzyme-specific metabolites of CYP1A2, 2B6, and 3A were quantified using LC–MS/MS analysis. The mRNA levels of CYP1A2, CYP2B6, and CYP3A4/5 were quantified from cell lysates using a Quantigene High Volume Kit purchased from Genospectra (Fremont, CA) as described previously [9].

Results

CYP inhibition

The highest concentration of tanespimycin tested in the inhibition assay was 60 μM due to solubility limitations. All positive controls inhibited the activities of their respective CYP enzymes to predefined acceptable levels (data not shown). The inhibitory effects of tanespimycin on selective probe activities of individual CYP enzymes in HLM are summarized in Table 1. When tanespimycin was co-incubated with probe substrates, it inhibited CYP2C19 and CYP3A4/5 with IC50 values of 5.6 μM (CYP2C19 and $CYP3A_T$) and 2.0 μM ($CYP3A_M$). It also weakly inhibited CYP2C8, CYP2C9, and CYP2D6 with IC50 values of 29,

Table 1 IC50 values of tanespimycin and 17-AG in the inhibition of enzyme-specific activities of 9 CYP enzymes in HLM

CYP enzyme	IC50 (μM)			
	Tanespimycin		17-AG	
	No preincubation	Pre-incubation	No preincubation	Pre-incubation
CYP1A2	>60	>60	>30	>30
CYP2A6	>60	>60	>30	>30
CYP2B6	>60	46	>30	>30
CYP2C8	29	32	>30	>30
CYP2C9	42	50	>30	>30
CYP2C19	5.6	8.2	3.2	5.1
CYP2D6	37	34	>30	>30
CYP2E1	>60	>60	>30	>30
$CYP3A_M$	2.0	0.56	6.4	4.7
$CYP3A_T$	5.6	1.3	8.0	8.3

$CYP3A_M$: CYP3A4/5 activity determined by midazolam 1′-hydroxylation; $CYP3A_T$: CYP3A4/5 activity determined by testosterone 6β-hydroxylation

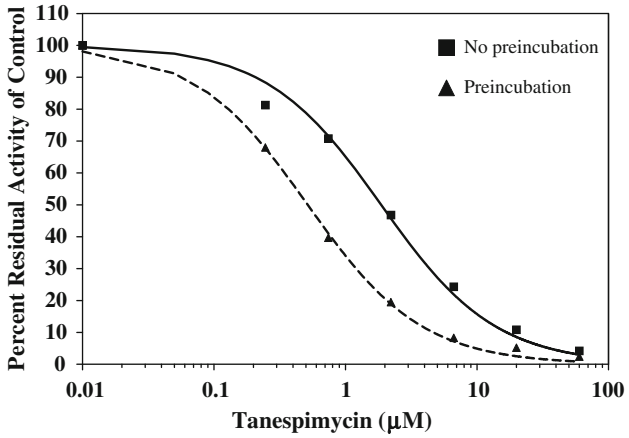

Fig. 2 IC50 *curves* for the inhibition of CYP3A4/5-mediated midazolam 1′-hydroxylase activity by tanespimycin with or without 30 min preincubation with HLM in the presence of NADPH

42, and 37 μM, respectively. There is no significant inhibitory of CYP1A2, 2A6, 2B6, or 2E1 up to 60 μM tanespimycin. Upon 30 min pre-incubation of tanespimycin with HLM in the presence of NADPH, a fourfold left shift of IC50 was observed in the inhibition of both probe substrate activities of CYP3A4/5. The IC50 curves of tanespimycin effects on $CYP3A_M$ with or without preincubation are shown in Fig. 2.

The highest concentration of 17-AG tested in the inhibition assay was 30 μM, also due to solubility limitations in the assay conditions. The inhibitory effects of 17-AG on selective probe activities of individual CYP enzymes in HLM are summarized in Table 1. Similar to tanespimycin, without preincubation, 17-AG inhibited CYP2C19 and CYP3A4/5 with IC50 values of 3.2 (CYP2C19), 6.4 ($CYP3A_M$), and 8.0 μM ($CYP3A_T$), however, it did not inhibit the activities of other CYP enzymes to appreciable extent up to 30 μM. Different from tanespimycin, upon 30 min pre-incubation of 17-AG with HLM in the presence of NADPH, no appreciable shift of IC50 was observed in the inhibition of the probe substrate activities of any enzyme.

CYP induction

Induction results for tanespimycin and 17-AG assays were averaged from triplicate samples and compared to the corresponding adjusted positive controls. The percent positive control induction level is arbitrarily set at 100% for the positive control specific to a given enzyme. The effects of tanespimycin and 17-AG on CYP activities are shown in Table 2, and their effects on the mRNA levels of CYP enzymes are shown in Table 3. Increases in enzyme activity or mRNA levels ≥40% of the respective positive control samples are considered an indication of demonstrable induction [10, 11].

The enzymes activities after treatment with tanespimycin and 17-AG were all less than 40% of their respective positive control treatments. In addition, the mRNA levels of CYP1A2, CYP2B6, and CYP3A4 were mostly below 40% of positive control after treatment with tanespimycin and 17-AG, except for CYP2B6 and CYP3A4 mRNA levels in incubations with hepatocytes from one donor (Hu1082), where >20 μM tanespimycin treatment resulted in mRNA levels of >50% of positive control. Overall, the results from this study suggest that tanespimycin and 17-AG, at the concentrations examined (2–40 μM and 1–20 μM, respectively), are not significant inducers of CYP1A2, CYP2B6, and CYP3A4.

Table 2 Summary of enzyme activity (% adjusted positive control) after treatment with tanespimycin and 17-AG

Treatment	CYP1A2			CYP2B6			CYP3A4/5		
	Hu1081	Hu1082	Hu1083	Hu1081	Hu1082	Hu1083	Hu1081	Hu1082	Hu1083
3-MC (2 μM)	100	100	100	1.2	3.1	0.97	−3.0	7.1	4.5
PB (1,000 μM)	3.9	7.5	6.2	100	100	100	54.2	54.4	52.5
RIF (10 μM)	3.0	3.6	3.8	25.1	41.9	11.1	100	100	100
Tanespimycin (2 μM)	−0.47	0.41	−0.79	−0.73	3.8	−0.12	−2.9	−5.5	0.46
Tanespimycin (20 μM)	−0.29	−0.16	−0.96	2.3	10.4	1.9	−1.6	−8.5	3.2
Tanespimycin (30 μM)	−0.18	0.53	0.05	4.4	14.7	7.3	−4.2	−6.5	3.6
Tanespimycin (40 μM)	−0.14	0.86	−0.58	4.3	15.9	5.0	−2.5	−6.0	0.83
17-AG (1 μM)	−0.43	−0.44	−0.85	−1.7	−0.55	−1.0	−7.1	−9.7	0.31
17-AG (3 μM)	−0.61	0.26	−1.1	−1.5	1.3	−1.1	−7.9	−7.2	0.26
17-AG (10 μM)	−0.24	0.87	−0.27	0.17	6.9	0.76	−5.3	−6.1	1.9
17-AG (20 μM)	0.52	0.79	0.39	4.5	9.7	4.9	2.6	−3.1	5.5

Table 3 Summary of mRNA content (% adjusted positive control) after treatment with tanespimycin and 17-AG

Treatment	CYP1A2			CYP2B6			CYP3A4		
	Hu1081	Hu1082	Hu1083	Hu1081	Hu1082	Hu1083	Hu1081	Hu1082	Hu1083
3-MC (2 μM)	100	100	100	0.76	35.5	0.50	−1.6	33.7	−1.5
PB (1,000 μM)	0.00	0.67	0.13	100	100	100	78.3	202	70.1
RIF (10 μM)	0.07	0.26	0.34	35.3	39.2	22.7	100	100	100
Tanespimycin (2 μM)	−0.11	−0.09	−0.07	3.6	10.8	6.3	9.0	13.0	9.9
Tanespimycin (20 μM)	−0.12	−0.10	−0.15	24.5	62.4	20.7	25.6	52.9	17.4
Tanespimycin (30 μM)	−0.12	−0.11	−0.15	26.1	61.3	22.1	23.9	39.2	12.7
Tanespimycin (40 μM)	−0.12	−0.11	−0.15	25.3	56.7	14.8	17.0	24.7	5.2
17-AG (1 μM)	−0.09	−0.05	0.00	−1.0	2.6	1.9	−0.31	0.28	−1.7
17-AG (3 μM)	−0.10	−0.05	−0.06	2.4	10.7	3.6	12.2	16.5	6.4
17-AG (10 μM)	−0.09	−0.10	−0.11	7.3	26.6	10.0	24.6	32.6	22.2
17-AG (20 μM)	−0.12	−0.11	−0.13	12.6	42.9	13.2	15.6	34.9	15.6

Discussion

Even after years of breakthrough biological research toward the understanding of cancer biology, and generations of revolutionary drug discovery against different types of malignant tumors, the quest continues to find a cure for most cancers. Most malignant cancers eventually develop resistance toward chemotherapies including cytotoxics and targeted agents alike. Combination of drugs with different mechanism of action is necessary to delay the progression of these malignancies. The chaperone protein, Hsp90, is believed to play a central role in many processes involving multiple oncogenic proteins, thus inhibition of Hsp90 has potential to achieve better efficacy in combination with various targeted agents. As many small molecule chemotherapeutics are metabolized by drug-metabolizing enzymes, such as cytochrome P450s, it is important to understand the potential of an Hsp90 inhibitor to inhibit CYPs to avoid undesirable drug interactions.

Tanespimycin is one of the first generation Hsp90 inhibitors. In cancer patients after a 1-h intravenous infusion of 295 mg/m^2 tanespimycin, the mean maximum total plasma concentration of tanespimycin and 17-AG were 17.3 and 5.9 μM, respectively [12]. Tanespimycin and 17-AG are both high plasma protein bound with unbound fractions approximately 5.7 and 7.8%, respectively [12]. The total concentrations are comparable to or exceed the IC50 values determined for the inhibition of CYP3A4/5 and CYP2C19. Even after the protein binding correction, the free plasma concentrations are still relevant because the I/IC50 value for tanespimycin is still over 0.1. Furthermore, the inhibition of CYP3A4/5 by tanespimycin is time-dependent. Therefore, it is possible that pharmacokinetic interactions can occur between tanespimycin and drugs that are metabolized by CYP3A4/5 and CYP2C19.

The mechanism of the time-dependent inhibition of CYP3A4/5 by tanespimycin is unknown. Tanespimycin is metabolized by CYP3A4/5 to form a reactive epoxide, a diol, and 17-AG [3]. In addition, tanespimycin and 17-AG can both undergo NADPH-cytochrome P450 reductase mediated one-electron reduction leading to reactive semiquinones [13]. Both the epoxide and the semiquinones could potentially react with electrophilic moieties within CYP3A4/5, and consequently result in the inactivation of CYP3A4/5. Because the inhibition of 17-AG was not time-dependent, the epoxide metabolite of tanespimycin could be responsible for the inactivation of CYP3A4/5. Although there was precedence for alkene epoxide to inactivate CYPs [14], additional experiments are needed to test the involvement of tanespimycin epoxide in the inactivation of CYP3A4/5.

In primary human hepatocytes, both tanespimycin and 17-AG at clinically relevant concentrations did not induce the enzyme activities of CYP1A2, CYP2B6, and CYP3A4/5. However, there were significant elevations of mRNA levels of CYP2B6 and CYP3A4 by high concentrations of tanespimycin in hepatocytes from one donor. In Hu1802, the elevation of mRNA levels did not translate into higher enzymatic activities of CYP3A4/5 and CYP2B6. The apparent lack of correlation between mRNA and enzyme activity of CYP3A4 could be explained by the potential inactivation of CYP3A4/5 by tanespimycin, but this is not true for CYP2B6 because tanespimycin had only minimal if any inhibition of CYP2B6 activities. In addition, a literature survey suggests downregulation rather than induction of CYP2B by inhibition of Hsp90. Hsp90 is proposed to form a complex with CAR to facilitate its nuclear translocation, and the inhibition of Hsp90 could disrupt CAR translocation and subsequently downregulates CYP2B transcription. In fact, the inhibition of Hsp90 by a

structurally similar Hsp90 inhibitor, geldanamycin, led to reduced nuclear translocation of CAR and consequently reduced induction of *cyp2b10* by phenobarbital in mouse hepatocytes [15]. Therefore, the mRNA level elevation in one out of three donor hepatocytes may not be significant.

Conclusion

In summary, the administration of tanespimycin is not expected to alter the exposure of drugs that are substrates of CYP1A2, CYP2A6, and CYP2E1. However, pharmacokinetic interactions are possible between tanespimycin and co-administered drugs that are substrates of CYP3A4/5 and CYP2C19 because tanespimycin and its active metabolite, 17-AG, are both inhibitors of CYP3A4/5 and CYP2C19 at clinically relevant concentrations. Neither tanespimycin nor 17-AG is an inducer of CYP1A2, CYP2B6, and CYP3A4/5, thus pharmacokinetic interactions due to the induction of these enzymes are unlikely. Based on these results, clinical drug–drug interaction studies are recommended between tanespimycin and probe substrates of CYP3A4/5 and/or CYP2C19.

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Conflict of interest All authors are employees of Bristol–Myers Squibb which investigates tanespimycin as a potential treatment of cancer.

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