Melanoma-specific ferrocene esters of the fungal cytotoxin illudin M

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The unfavorable therapeutic index of the fungal cytotoxin illudin M was to be improved by covalent attachment of the redox modulator and phenyl isobiostere ferrocene. Esters of illudin M with ferrocenoic and 1,1′-ferrocenedioic acid were prepared, structurally characterised (X-ray), and tested for cytotoxicity [MTT assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], induction of apoptosis (TUNEL assay; western blotting for caspase-9), and tumor specificity in cells of human HL-60 leukemia, human 518A2 melanoma, and in nonmalignant human foreskin fibroblasts. The diester of illudin M with 1,1′-ferrocenedioic acid was distinctly more antiproliferative and apoptosis inducing in the melanoma cells [half maximal inhibitory concentration, $IC_{50}(48\,h) = 0.4 \pm 0.1\,\mu mol/l$] than in the HL-60 cells [$IC_{50}(48\,h) = 3.0 \pm 1.6\,\mu mol/l$] and in the nonmalignant fibroblasts [$IC_{50}(48\,h) = 3.7 \pm 1.9\,\mu mol/l$]. This corresponds to a doubling of the therapeutic index with respect to illudin M. The monoester of illudin M with ferrocenoic acid was nine times less efficacious in the cancer cells, when compared with the diester. In conclusion, the ferrocene diminishes the general toxicity of the illudin M moiety and increases its cell line specificity. The bis(illudinyl M) 1,1′-ferrocenedioate presumably operates by a synergistic, two-pronged attack on its molecular targets. *Anti-Cancer Drugs* 20:676–681 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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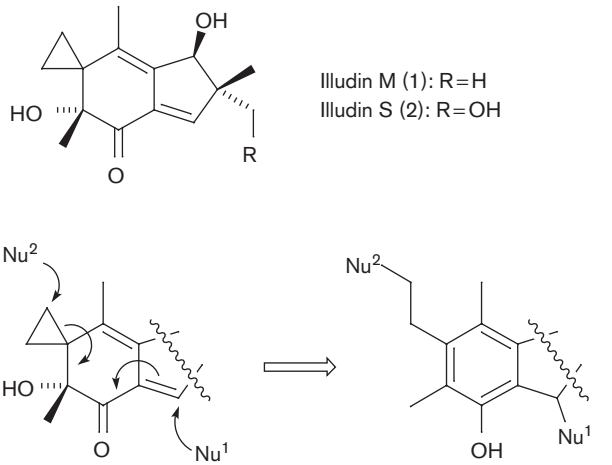
Introduction

The sesquiterpene illudin M (1) was first isolated from the culture medium of *Omphalotus olearius* mushrooms, together with illudin S (2) [1,2]. After reduction of their enone moieties by NADPH or glutathione (Nu^1), both compounds alkylate DNA, RNA, and proteins (Nu^2) through ring opening of the spirocyclopropane, and therefore eventually lead to apoptotic cell death (Fig. 1) [3–5]. Although the illudins are highly efficacious against various tumors, their extreme cytotoxicity has prevented clinical applications [6–9]. Kinder *et al.* [10] and McMorris *et al.* [11,12] reported semisynthetic illudin derivatives with reduced toxicity and activity, but improved therapeutic indices. Hydroxymethylacylfulvene (irofulven) [13] has been in phase II clinical trials for a number of cancers including hormone refractory prostate, ovarian, pancreatic, renal, colorectal, lung, and breast cancer. However, except for some prostate and pancreatic cancers it proved largely ineffective while hampered with severe side effects, such as prolonged reversible neutropenia at low and retinotoxicity at high doses [14–21].

In a preceding study, we disclosed a simplified procedure for the isolation of illudin M from culture broths of *O. olearius* and some illudinyl M esters that were as active as illudin M in HT-29 colon and Panc-1 pancreatic carcinoma cells while generally less toxic [22]. Now, we report on two ferrocene esters of 1, which are highly specific for 518A2 melanoma cells, whereas less noxious to leukemia cells and nonmalignant fibroblasts.

Fig. 1



Natural cytotoxins illudin M (1) and illudin S (2) and their general two-stage reaction with bionucleophiles Nu.

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Materials and methods

Illudinyl M esters

Illudin M (1) was extracted from the culture medium of *O. olearius* and esterified as previously published for docosahexaenoic acid [22]. The respective ferrocen (edi)oic acid was first treated with Et_3N and 2,4, 6-trichlorobenzoyl chloride to give a mixed anhydride within *ca.* 20 min. This was treated with illudin M and 4-(*N*-dimethylamino)pyridine to afford the corresponding new esters 3a and 3b for which satisfactory microanalyses (C, ± 0.2; H, ± 0.1) were obtained after purification. NMR spectra were recorded in $CDCl_3$.

Bis(illudinyl M) 1,1′-ferrocenedicarboxylate (3a)

Yield 47%; brown solid, mp 73°C; υ_{max}/cm^{-1}: 1698, 1657, 1607; ^{1}H NMR (300 MHz) δ 0.3–0.4 (2 H, m), 0.7–0.9 (4 H, m), 1.0–1.2 (8 H, m), 1.36 (6 H, s), 1.56 (6 H, s), 3.58 (2 H, s), 4.3–4.4 (4 H, m), 4.7–4.8 (4 H, m), 5.73 (2 H, s), 6.53 (2 H, s); ^{13}C NMR (75 MHz) δ 5.9, 8.8, 14.9, 20.7, 24.6, 26.6, 31.4, 48.8, 71.2, 72.8, 72.9, 75.9, 78.8, 133.5, 135.2, 135.5, 146.3, 170.0, 199.7; *m/z* (EI) 734 (4) [M^+], 541 (6), 504 (7), 274 (100).

Illudinyl M ferrocenecarboxylate (3b)

Yield 59%; red brown solid, mp 145°C; υ_{max}/cm^{-1}: 1713, 1696, 1659, 1599; ^{1}H NMR (300 MHz) δ 0.3–0.4 (1 H, m), 0.8–1.0 (2 H, m), 1.0–1.1 (1 H, m), 1.15 (3 H, s), 1.19 (3 H, s), 1.40 (3 H, s), 1.59 (3 H, s), 3.58 (1 H, s), 4.17 (5 H, s), 4.3–4.4 (2 H, m), 4.7–4.8 (2 H, m), 5.77 (1 H, s), 6.56 (1 H, s); ^{13}C NMR (75 MHz) δ 5.9, 8.8, 14.9, 20.8, 24.6, 26.6, 31.4, 48.9, 69.6, 70.1, 71.2, 76.0, 78.5, 133.5, 135.3, 135.5, 146.5, 171.3, 200.0; *m/z* (EI) 461 (5) [M^+], 335 (11), 230 (100). Crystal data: $C_{26}H_{29}FeO_4$, $M = 461.34$, monoclinic, space group C2, $a = 21.555(4)$, $b = 7.4268(15)$, $c = 15.631(3)$ Å, $\alpha = \gamma = 90°$, $\beta = 115.75(3)°$, $V = 2253.9(8)$ Å^3, $Z = 4$, $\lambda = 0.71073$ Å, $\mu = 0.699\,mm^{-1}$; 4053 unique reflections; refinement to convergence on F^2 gave $R_w = 0.1047$ (all unique data), GOF = 1.011. The file CCDC-730531 contains the supplementary crystallographic data of this study, which can be obtained free of charge from The Cambridge Crystallographic Data Centre at *www.ccdc.cam.ac.uk/data_request/cif*.

Cell lines and culture conditions

The human HL-60 cells were obtained from the German National Resource Center for Biological Material (DSMZ, Braunschweig, Germany), the human 518A2 melanoma cells from the Department of Oncology and Hematology of the Martin Luther University, Halle, Germany, and the human foreskin (HF) fibroblasts from the University Hospital Erlangen, Germany. The HL-60 cells were incubated in RPMI (Roswell Park Memorial Institute) media 1640 with 10% foetal bovine serum, 1% antibiotic–antimycotic and 0.5% gentamycin (all Gibco, Egenstein, Germany). The 518A2 melanoma and HF cells were cultured in Dulbecco's modified Eagle medium (Gibco, Egenstein, Germany) with the same additions, the fibroblasts in Dulbecco's modified Eagle medium with 10% foetal bovine serum, 10^7 U/l penicillin and 10 mg/l streptomycin.

Cell proliferation assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (ABCR) was used to identify viable cells that reduce it to a violet formazan [23]. HL-60 cells (5×10^5 cells/ml), the adherent 518A2 cells (17×10^5 cells/cm^2), and HF cells (5×10^4 cells/ml) were seeded and cultured for 24 h. Incubation (5% CO_2, 95% humidity, 37°C) following treatment with the test compounds (dilution series ranging from 0.001 to 50 µmol/l in phosphate saline buffer) was continued for 24 and 48 h, respectively. Blank and solvent controls were treated identically. MTT in phosphate-buffered saline (5 mg/ml) was added to a final concentration of 0.5 mg/ml. After 2 h, the formazan precipitate was dissolved in 10% sodium dodecylsulfate in dimethyl sulfoxide containing 0.6% acetic acid in the case of the HL-60 cells. For the adherent cells, the microplates were swiftly turned to discard the medium before adding the mixture. After 12 h incubation, the absorbance at wavelength 570–630 nm was measured with an automatic microplate reader (MWG-Biotech, Milton Keynes, UK). All experiments were carried out in triplicate; the percentage of viable cells quoted was calculated as the mean ± SD with respect to the controls set to 100%.

Immunoblotting with caspase-9 antibodies

518A2 melanoma cells were incubated with the test compounds at their half maximal inhibitory concentration (IC_{50}). At specified time intervals, the detached cells in the supernatant plus the trypsinated cells were pooled, and after centrifugation suspended in lysis buffer [100 µl, 50 mmol/l Tris–HCl, 150 mmol/l NaCl, 1% Triton X-100, 2% Protease Inhibitor Cocktail Set III (Calbiochem, Bad Soden, Germany)], vortexed and incubated on ice for 15 min. The proteins were separated on a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (70 µg per pocket) and blotted on a polyvinylidene fluoride membrane blocked with 10% milk powder. After sequential incubation with the primary (Calbiochem, 1 : 10.000 in AP-T) and secondary antibodies (conjugated with horseradish peroxidase; Calbiochem, 1 : 10.000 in AP-T), visualization was conducted with Roti-Lumin (Carl Roth, Karlsruhe, Germany) and Amersham Hyperfilm ECL (GE Healthcare, Muenchen, Germany).

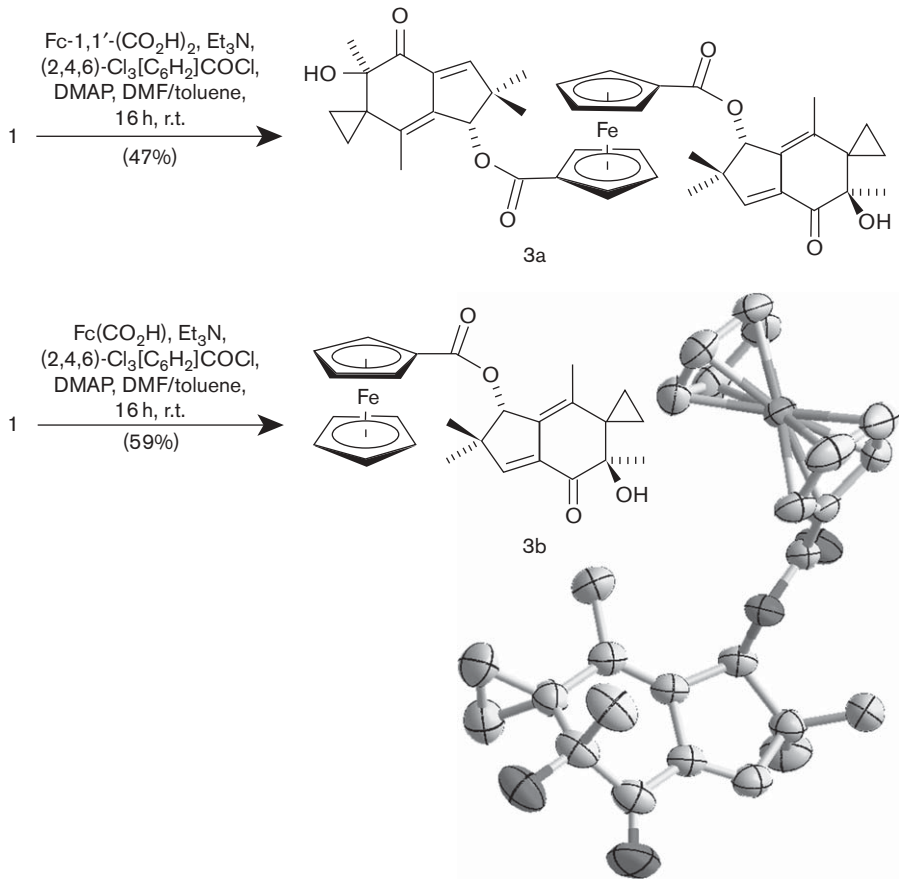
Results and discussion

Melanoma cells are known to overexpress melanotransferrin (p97), a high-affinity iron-binding protein, which is believed to play a role in cell proliferation rather than in cellular Fe uptake [24,25]. A few derivatives of ferrocene, a nontoxic though bioactive iron complex [26], with enhanced antiproliferative activity in melanoma cells

have already been reported [27,28]. With the intention to enlarge the therapeutic index and the cell line specificity of illudin M (1), new esters with 1,1′-ferrocenedioic acid (3a) and ferrocenoic acid (3b) were prepared. The first X-ray single crystal structure analysis of an illudin M derivative was also carried out for the monoester 3b (Fig. 2). Both esters were tested for their antiproliferative activities in cells of human HL-60 leukemia and 518A2 melanoma, and also in nonmalignant HF fibroblasts (Table 1).

The esters 3a and 3b were less cytotoxic than illudin M in MTT assays against the cancer cells. However, the diester 3a was still distinctly antiproliferative in the melanoma cells at $IC_{50}(48\,h) \approx 0.42\,\mu mol/l$. 518A2 melanoma cells are known to be rich in antiapoptotic bcl-2 protein [29] and mTOR [30], a downstream kinase of the PI3K/Akt signaling pathway that also acts as a sensor of the cellular redox status and which is thought to contribute to the chemoresistance of these cells, for example against cisplatin [$IC_{50}(48\,h) > 10\,\mu mol/l$]. In the p53-null HL-60 leukemia cells, the esters 3a and 3b were about seven times less active. This cancer cell line specificity is unusual as to its magnitude and inverse to that of most other anticancer drugs that are more active in HL-60 cells. Diester 3a was also considerably less active in the nonmalignant fibroblasts [$IC_{50}(48\,h) \approx 3\,\mu mol/l$] than in the melanoma cells by a factor of eight which corresponds to a doubling of the therapeutic index with respect to illudin M. This improvement originates at least in part from the ferrocene shielding the enone group against attack of glutathione. Using UV spectroscopy to monitor the decay of the enone band of compounds 1 and 3a and 3b at 330 nm in the presence of glutathione showed a stabilization of conjugates 3a and 3b compared with illudin M (Fig. 3). A cleavage of the ester bond over the time of observation (2 h) can be ruled out as neither free illudin M nor free ferrocen(edi)oic acids were detectable by TLC and HPLC. In contrast, diester 3a was not just two-fold, but nine-fold more efficacious

Fig. 2



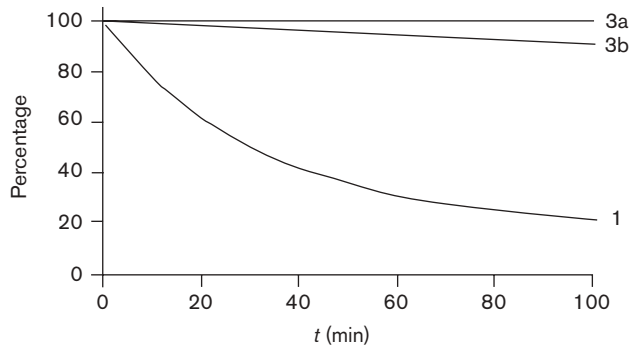
Syntheses of illudinyl M (1) esters bis(illudinyl M) 1,1′-ferrocenedicarboxylate (3a) and illudinyl M ferrocenecarboxylate (3b) and the molecular structure of ester 3b as obtained from an X-ray single crystal structure analysis (ORTEP representation, 50% probability ellipsoids); hydrogen atoms are omitted (CCDC 730531). DMAP, 4-(*N*-dimethylamino)pyridine. DMF, dimethyl formamide; r.t., room temperature; ORTEP, Oak Ridge Thermal Ellipsoid Plot.

Table 1 IC_{50} values[a] of illudin M (1) and esters 3a and 3b in cells of human HL-60 leukemia, human 518A2 melanoma, and HF fibroblasts

Compound	IC_{50}(μmol/l)[a]			Ratio IC_{50}(HF)/ IC_{50}(518A2)
	HL-60	518A2	HF	
1	0.02 ± 0.01	0.03 ± 0.02	0.13 ± 0.03	4.3
3a	3.0 ± 1.6	0.42 ± 0.08	3.66 ± 1.90	8.7
3b	28 ± 5.3	3.6 ± 0.5	9.51 ± 2.49	2.6

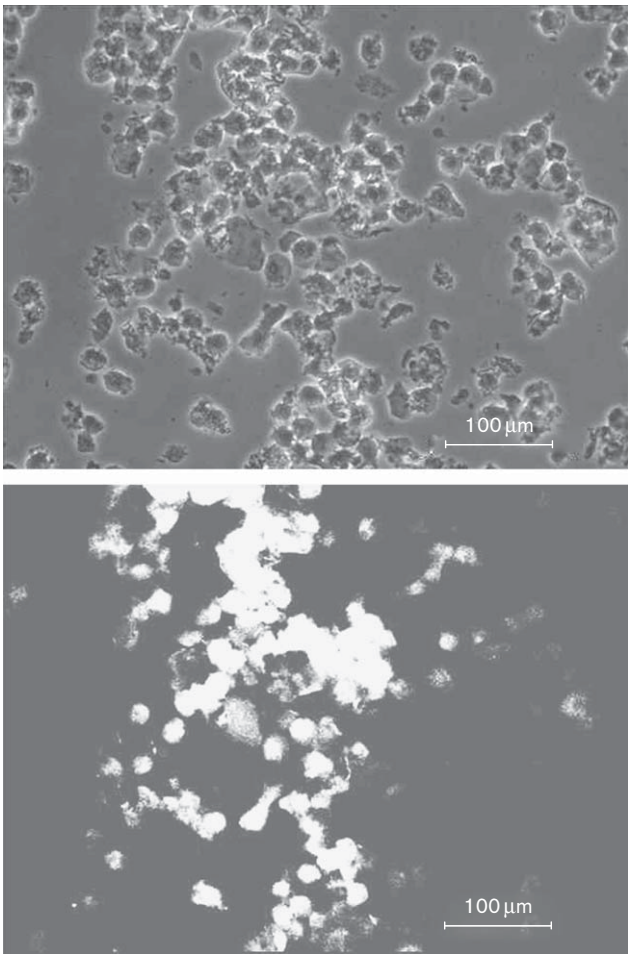
3a, bis(illudinyl M) 1,1′-ferrocenedicarboxylate; 3b, illudinyl M ferrocenecarboxylate; HF, human foreskin fibroblasts; IC_{50}, half maximal inhibitory concentration. [a]Values are derived from concentration–response curves obtained by measuring the percentage of vital cells relative to untreated controls (100%) after 48 h exposure to test compounds in the MTT assay (3–(4,5–dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). Values represent mean ± SD of three independent experiments in duplicate.

Fig. 3



Temporal progress of the ultraviolet absorption at 330 nm (enone group) of mixtures of 200 μmol/l of illudin M (1) or its esters bis(illudinyl M) 1,1′-ferrocenedicarboxylate (3a) and illudinyl M ferrocenecarboxylate (3b) and 6.5 mmol/l glutathione in phosphate-buffered saline, measured every 30 s. Initial absorption was set to 100% in each case. Relative decay rates were calculated as 1.8×10^{-2} for 1, 2×10^{-5} for 3a, and 9×10^{-4} for 3b.

Fig. 4



518A2 melanoma cells exposed to bis(illudinyl M) 1,1′-ferrocenedicarboxylate (3a) (10 μmol/l) for 15 h and then tested with the TUNEL assay. Top panel: sample in the visible channel showig all cells; bottom panel: green fluorescence image (excitation: 450–500 nm; detection: 515–565 nm) of the same sample, bright fluorescent spots indicate apoptotic cells.

in both the cancer cell lines than the monoester 3b. This suggests that the two illudin residues in 3a operate by a synergistic, two-pronged attack on their molecular targets.

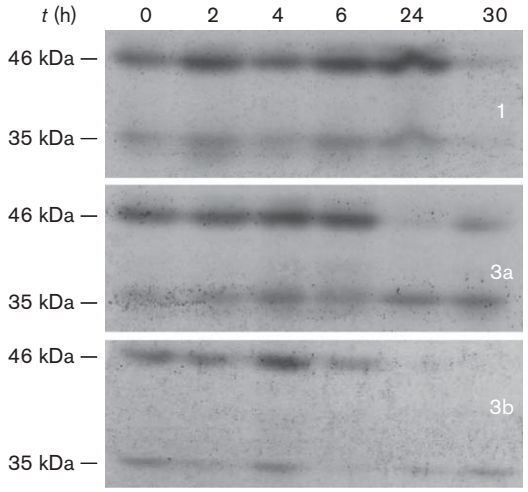
Illudin M and its esters 3a and 3b induced apoptosis in both cancer cell lines as ascertained by TUNEL assays that allow the detection of late stages of apoptosis by labeling the 3′-OH ends of typical DNA fragments with fluorescein-tagged nucleotides [31]. Fluorescence microscopy after 15 h exposure to the test compounds revealed *ca.* 50% apoptotic melanoma cells for compounds 1 and 3a (Fig 4) but only *ca.* 20% for monoester 3b. Only *ca.* 25% apoptotic HL-60 cells were found after 15 h exposure to esters 3a and 3b compared with 50% for illudin M.

Illudin derivatives, such as irofulven, are known to induce apoptosis not through the intrinsic caspase-9-dependent pathway, but by a mechanism related to the activation of JNK and ERK kinases, regardless of the p53 and bcl-2 status of the cells [32,33]. Immunoblotting for (pro)caspase-9 in bcl-2 rich 518A2 melanoma cells treated with illudin M or its esters 3a and 3b showed a similar picture. Illudin M caused an increase of procaspase-9 and caspase-9 protracted over 24 h. This slow conversion of the procaspase into the active caspase is unlikely to be decisive for apoptosis. Although the esters 3a and 3b cleaved the procaspase-9 more rapidly (4–6 h), these, also, led to the persistence of active caspase-9 for more than 24 h (Fig 5).

Conclusion and outlook

Bis(illudinyl M) 1,1′-ferrocenedioate (3a) is far less toxic to nonmalignant fibroblasts, more cancer selective and more cell line-specific than the parent illudin M. In 518A2 melanoma cells, it retained an antiproliferative

Fig. 5



Time-dependent processing of procaspase-9 (*ca.* 46 kDa) and caspase-9 (*ca.* 35 kDa) in 518A2 melanoma cells treated with compounds illudin M (1) and bis(illudinyl M) 1,1′-ferrocenedicarboxylate (3a) and illudinyl M ferrocenecarboxylate (3b) for up to 30 h. ECL visualization of lysates immunoblotted with primary/secondary antibodies to caspase-9 from Calbiochem. Data are representative of three independent experiments.

effect at submicromolar IC_{50}(48 h) concentrations. The fact that two illudinyl residues on a central ferrocene hub gave rise to a synergistic overadditive boost in anticancer efficacy renders similar conjugates with other metal fragments attractive. Particularly interesting are metals with a genuine anticancer activity of their own such as Ru or Ga. By using the α-hydroxy ketone group of illudin M as a chelating *O,O*-ligand, complexes such as tris(illudinyl M)Ga(III) should be readily accessible. Respective investigations are currently under way.

Acknowledgement

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