Preclinical evaluation of properties of a new targeted cytotoxic somatostatin analog, AN-162 (AEZS-124), and its effects on tumor growth inhibition

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In view of findings that various tumors express receptors for somatostatin, a new targeted cytotoxic analog of somatostatin, AN-162 (AEZS-124), consisting of doxorubicin linked through glutaric acid to the somatostatin octapeptide RC-121 was developed in our laboratory. We studied the toxicity *in vivo* and the effect of AN-162 on growth of the MDA-MB-231 estrogen-independent human breast cancer cell line xenografted into nude mice. AN-162 induced significant tumor growth inhibition compared with the control and the group treated with doxorubicin in equimolar doses. We also evaluated the stability of AN-162 in various sera *in vitro*, as this conjugate is susceptible to hydrolysis by serum carboxylesterase enzymes in the circulation. This study shows for the first time that AN-162 is a safe and effective compound for the treatment of experimental breast cancer. Our findings support the concept of targeted chemotherapy based on cytotoxic peptide analog AN-162 for the treatment of breast cancers and other cancers expressing somatostatin receptors. *Anti-Cancer Drugs* **20:553–558 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

The cytotoxic agent doxorubicin (DOX) is one of the most widely used chemotherapeutic drugs. The therapeutic effect is mainly because of the ability of DOX to intercalate into the DNA double helix and inhibit topoisomerase II. However, toxic effects of DOX such as cardiomyopathy and myelosuppression might occur during or after the treatment. The side effects of anthracyclines may require the interruption of therapy or even complete termination of the treatment. It is also known that intrinsic or acquired resistance to DOX reduces the response to DOX therapy. Many approaches, with varying results, have been tried to decrease the side effects by modifying the mode of delivery of DOX to the tumor cells [1–6]. In our laboratory, we developed a series of targeted cytotoxic peptide conjugates in which DOX is linked through glutaric acid to analogs of bombesin, luteinizing hormone-releasing hormone (LHRH), or somatostatin (SST). The targeted cytotoxic analog of LHRH [AN-152 (AEZS-108)] is already in phase II clinical trials. A high active derivate of DOX, 2-pyrrolino-DOX (AN-201), was also synthesized in our laboratory and coupled through the same linkage to these peptides [7–10]. The targeted cytotoxic SST analog linked to AN-201 is AN-238 [10]. In many earlier studies, we showed that these cytotoxic compounds are more efficacious in inhibiting tumor growth in nude mice bearing xenografts of various human cancers and less toxic than the cytotoxic radicals [6,11–18]. Various preclinical studies showed the expression of SST receptors (sstr) in many human malignancies [11–18]. As AN-238 is not yet available and in view of the success of related LHRH analog AN-152 (AEZS-108) in phase I clinical trials, we consequently decided to investigate the cytotoxic analog of SST AN-162 (AEZS-124) consisting of SST octapeptide RC-121 linked to DOX (Fig. 1) [10]. The outcome of our approach based on targeted chemotherapy depends greatly on the delivery of AN-162 into the tumor tissue. Therefore, the binding ability of AN-162 to the sstr in sstr-positive tumor tissues and the stability of the conjugate in the circulation are important. In earlier studies, it was shown that serum carboxylesterase enzymes (CE) could hydrolyze cytotoxic conjugates up to 10 times faster in mouse serum than in human sera [19–21]. For further studies of AN-162, it is essential to know the stability of our cytotoxic compound in different

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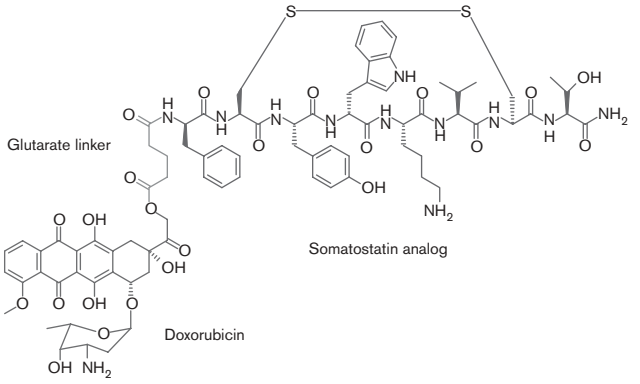
Fig. 1



Molecular structure of cytotoxic somatostatin analog AN-162. Cytotoxic radical doxorubicin is linked through the glutaric acid spacer to the octapeptide RC-121.

laboratory settings. To show the efficacy of AN-162, we first carried out a toxicity study to find the maximal tolerated dose (MTD) for this compound in nude mice. This was followed by a therapeutic study using MDA-MB-231 estrogen-independent breast cancer xenografts.

Materials and methods

Cell culture

The estrogen-independent breast cancer cell line MDA-MB-231 was obtained from the American Type Culture Collection (Manassas, Virginia, USA). Cells were cultured in the Dubecco's modified essential media (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin at 37°C and 5% CO_2 atmosphere. Chemicals, unless stated otherwise, were purchased from Sigma (St. Louis, Missouri, USA).

Analogs

Cytotoxic SST analog AN-162 (AEZS-124), first synthesized by our laboratory was provided for this study by the Aeterna Zentaris GmbH, Frankfurt am Main, Germany [10]. For intravenous (i.v.) injection of the animals AN-162 was disolved in 0.01N acetic acid and diluted with 5% mannitol.

SST analog RC-160 (vapreotide acetate) first synthesized by our laboratory was provided by the Genzyme (Cambridge, Massachusetts, USA). RC-160 is equivalent to RC-121 and was used instead of RC-121, which was not available for this study [22,23]. DOX was obtained from Chemtec Leuna GmbH (Leuna, Germany).

PCR

Total RNA was isolated and DNAse treated using the Macherey-Nagel NucleoSpin kit according to the manufacturer's instructions (Macherey-Nagel, Germany). The yield and quality of RNA samples were determined spectrophotometrically using 260 nm, and 260/280 and 260/230 nm ratios. Five hundred nanogram of RNA were reverse transcribed into cDNA by Moloney murine leukemia virus reverse transcriptase using random primers (Promega, Wisconsin, USA) in a final volume of 20 µl. A negative control, with no reverse transcriptase added, was also included. cDNA (1 µl) was amplified in a 25 µl of solution containing 1.5 mmol/l $MgCl_2$, 1 × PCR buffer (Invitrogen, California, USA), 0.2 mmol/l of each deoxynucleotide (Promega), 1 U of Platinum Taq DNA polymerase (Invitrogen) and 0.25 µmol/l of each primers. The integrity of cDNA was tested by reverse-transcription PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Samples were denatured for 2 min at 94°C and then subjected to 40 cycles of 94°C for 45 s, 60°C (sstr1, sstr2, sstr5, GAPDH), 62°C (sstr3) or 55°C (sstr4) for 30 s, then 72°C for 1 min and 30 s with a final extension of 10 min at 72°C. All PCR reactions were done in an Applied Biosystems 2700 thermal cycler (Applied Biosystems, California, USA). Ten microliters of each amplification reaction were electrophoretically separated on 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light. Gene-specific primers for sstr1, sstr3, and sstr5 were as described earlier [24]; primers for sstr2, sstr4, and GAPDH were designed with the *primer3_www.cgi v 0.2 program*.

Receptor binding assay

The binding characteristics of receptors for SST were determined on membrane fraction of MDA-MB-231 cancer cells (approximately 180 × 10^6 cells) as described earlier [25]. For in-vitro ligand competition assays, radioiodination of SST analog RC-160 and separation of the monoiodinated radioligand by high-performance liquid chromatography (HPLC) were carried out.

Animal experiments

Six-week-old female nude mice (Ncr nu/nu) were obtained from the National Cancer Institute (Bethesda, Maryland, USA). The animals were housed in sterile cages in a temperature-controlled room with a 12-h light/12-h dark schedule. Autoclaved chow and water *ad libitum* was fed. At the end of each experiment, the mice were killed under pentobarbital anesthesia (intraperitoneally), tumors were excised and necropsy was performed. The Institutional Animal Care and Use Committee reviewed the protocols of the animal experiments and gave full approval.

Toxicity study

Thirty female nude mice (Ncr nu/nu) were divided into six groups, each group consisting of five animals. The groups of animals were treated with AN-162 at 5, 3.5, and 2.5 µmol/kg and RC-160 at 5, 3.5, and 2.5 µmol/kg.

AN-162 and RC-160 were injected into the jugular vein in a volume of 200 µl/20 g. The i.v. injection was given under deep isoflurane anesthesia on days 1, 4, and 8. To prevent hypothermia during the recovery from anesthesia, a heating pad was provided. After injection, the mice were observed for 1 h recording noninvasive criteria of uncomfortability and toxicity (cyanosis, piloerection, motor function, agitation, humping, breathing) every 10 min continuously for 60 min. If death occurred, necropsy was performed to exclude, for example, major bleeding into the thorax because of failed procedure.

Therapy study

For the therapy study, 10×10^6 cells/mouse of the human estrogen-independent breast cancer cell line MDA-MB-231 were injected into the flanks of four female nude mice (Ncr nu/nu). The resulting tumors were harvested and minced into approximately 3 mm^3 pieces and transplanted into both flanks of female nude mice using a mini trocar. After the tumors reached approximately 32 mm^3, the mice bearing MDA-MB-231 xenografts were randomized into five groups, which received i.v. injections once a week for 4 weeks of AN-162 (2.5 µmol/kg) (animals $n = 10$; tumors $n = 19$), DOX (2.5 µmol/kg) (animals $n = 10$; tumors $n = 18$), SST analog RC-160 (2.5 µmol/kg) (animals $n = 8$; tumors $n = 13$), SST analog RC-160 (2.5 µmol/kg) plus DOX (2.5 µmol/kg) (original animal $n = 7$, but because of death of three mice, final animals $n = 4$; tumors $n = 7$); control group received solvent only (animals $n = 11$; tumors $n = 19$). The doses of AN-162 were equivalent to 1.45 mg/kg DOX. Tumor volume was measured by a microcalliper once a week and calculated using the formula: length × width × height × 0.5236. The weights of the mice were recorded weekly. Tumor doubling time was calculated using the formula: study duration/(log final tumor volume – log initial tumor volume)/$\log_2$.

High-performance liquid chromatography

Sample preparation

For collecting mouse blood samples, five animals were anesthetized with isoflurane and blood was drawn from the jugular vein. The whole blood samples were allowed to coagulate for 1 h in an incubator at 37°C in 95% air/5% CO_2 atmosphere, serum was separated by centrifugation and serum samples were pooled. Three human blood samples were drawn from male volunteers and processed as described earlier without pooling. DMEM cell culture media was obtained from the American Type Culture Collection and FBS was purchased from the Altana Biologicals (Lawrenceville, Georgia, USA). AN-162 was dissolved in 5% mannitol and 10 µl of solution containing 10 µg of AN-162 was added to 90 µl of the samples. Three different samples for each time point were incubated for 0, 10, and 30 min in mice sera; for 0, 30, and 60 min in human sera; 0, 60, and 180 min in FBS; and 0 and 120 min in DMEM plus 10% FBS in an incubator at 37°C. At the end of the incubation, perchloric acid 0.1N was added to stop hydrolysis and samples were quenched on ice for 15 min.

High-performance liquid chromatography analysis

Fifty microliters of the samples were applied on a Varian ProStar HPLC system (Palo Alto, California, USA). Seperation of the intact cytotoxic compound was carried out on a Vydac C_8 column (Hesperia, California, USA) (250 × 4.6 mm; pore size 300 Å; particle size 5 µm). The UV absorption was detected at 480 nm and the percentage of intact AN-162 was evaluated and half-life ($t_{1/2}$) of AN-162 was calculated. Linearity of the analysis method was proofed previously by running serial dilutions of AN-162 in mannitol 5% only.

Statistical analysis

For statistical analysis Student's two tailed *t*-test or analysis of variance group comparison (method of Bonferroni) was used. A P value of less than 0.05 was considered as significant.

Results

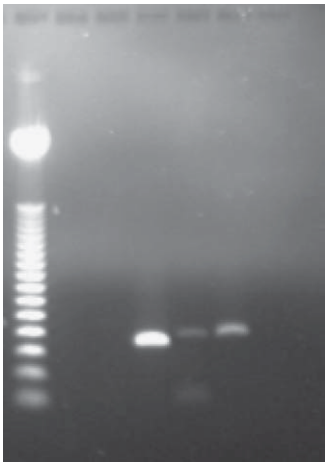
Toxicity study

In the groups of animals treated with 5 µmol/kg AN-162 or RC-160, all mice died after the first injection within a time frame of 20–40 min after administration. No mice were lost in the group treated with 3.5 µmol/kg of AN-162. In the group of animals treated with 3.5 µmol/kg RC-160, three mice died after the first injection (25, 30, and 40 min after administration) and two mice died after the second injection (25 min after administration). In the group of mice treated with 2.5 µmol/kg AN-162, one mouse died immediately after the first injection because of an accidental embolic event, but no other mice died. Similarly, in the group of animals treated with 2.5 µmol/kg RC-160, none of the mice died because of toxicity. After recovering from anesthesia, the mice showed symptoms such as cyanosis, piloerection, affected motor function, agitation, and humping after 20 min. These symptoms were because of toxicity of the substances, but not to failed procedure, such as embolic events where death occurs much earlier. Typically, death of mice because of toxicity was observed in a time frame of 20–40 min. All the mice, which died, presented severe respiratory symptoms, such as gasping. The MTD for AN-162 in nude mice was assumed to be 3.5 µmol/kg, whereas the MTD for RC-160 is 2.5 µmol/kg.

PCR

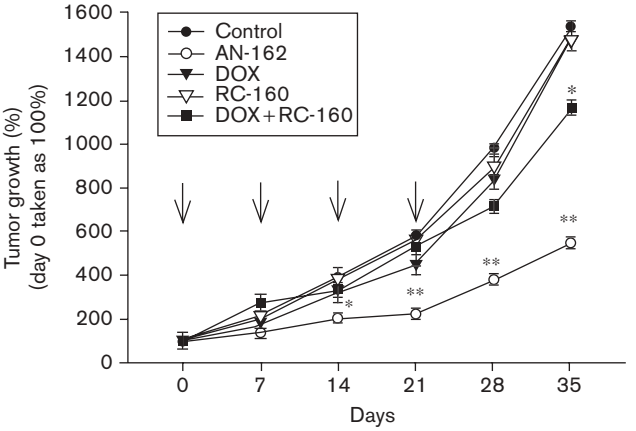
A strong expression of mRNA for sstr2 was found in MDA-MB-231. mRNA for sstr3 and sstr4 was also present in this cell line, but no expression of sstr1 and sstr5 at mRNA level was detected. Results of reverse-transcription PCR are summarized in Fig. 2.

Fig. 2



Reverse-transcription PCR gel electrophoresis of receptors of somatostatin (SST) from MDA-MB-231 human breast cancer cell line. M, 50-bp DNA size marker (Promega); N, negative control; 1, SST receptor (sstr)1; 2, sstr2; 3, sstr3; 4, sstr4; 5, sstr5.

Fig. 3



Effect of treatment with cytotoxic somatostatin analog AN-162 on the growth of MDA-MB-231 estrogen-independent human breast carcinoma cell line xenografted into nude mice (mean ± SE). AN-162, doxorubicin (DOX), somatostatin analog RC-160, and the unconjugated mixture of DOX plus RC-160 were administered at 2.5 μmol/kg. Arrows mark intravenous injections into the jugular vein. *$P<0.05$ (AN-162 vs. all groups), **$P<0.01$ (AN-162 vs. all other groups), analysis of variance tests. Three animals died in the RC-160 plus DOX group, but this group still contained seven tumors in four animals.

Binding assay

Using radiolabeled RC-160 as a radioligand, high affinity ($K_d = 5.64 \pm 1.45$ nmol/l) and low-capacity ($B_{max} = 356.9 \pm 62.7$ fmol/mg membrane protein) receptors for SST were detected in MDA-MB-231 cancer cells.

Therapy study

In the therapy study on animals with xenografted MDA-MB-231 estrogen-independent human breast cancer, the group treated with cytotoxic analog AN-162 showed a significant tumor growth inhibition after 14 days of therapy compared with the control group ($P < 0.01$) and the groups treated with equimolar doses of DOX ($P = 0.018$), SST analog RC-160 ($P < 0.01$) or the unconjugated mixture of DOX plus RC-160 ($P = 0.031$). In total, the mice received four i.v. injections. The difference in tumor growth between the group of animals treated with AN-162 and all other groups remained significant until the end of the study on day 35 (Fig. 3, Table 1). No significant difference in tumor growth was found between the control group and the groups of animals treated with DOX and RC-160. However, at the end of the study, the unconjugated combination of DOX plus RC-160 showed a small, but significant inhibition of tumor growth compared with the control group and the groups of animals treated with DOX alone or RC-160. The tumor doubling time in the group of animals treated with AN-162 was extended to 16.54 ± 1.46 days and was significantly different from that in the control group 9.4 ± 0.88 days and from doubling times in the other treatment groups (Table 1) The weights of the animals in the group treated with AN-162 showed no significant difference compared with those in the control group.

Table 1 Effect of therapy with cytotoxic analog of somatostatin AN-162 on the growth of MDA-MB-231 estrogen-independent human breast carcinoma xenografts

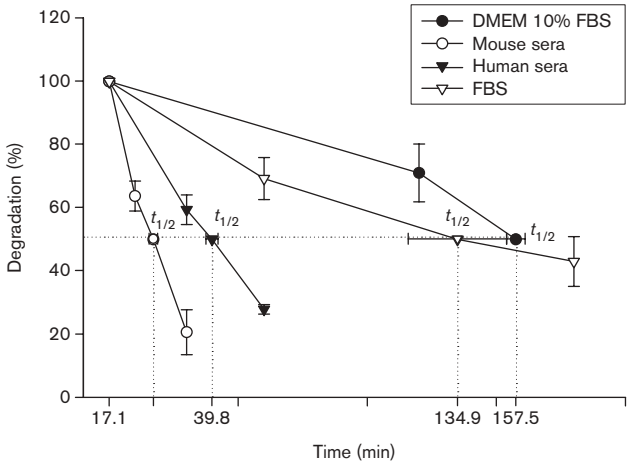
	Tumor growth (%) on day 35 (mean ± SE)	Tumor doubling time (days) (mean ± SE)
Control	1541 ± 26.04	9.40 ± 0.86
AN-162	543 ± 19.90**	16.54 ± 1.46*
DOX	1480 ± 53.27	11.99 ± 1.00
RC-160	1473 ± 43.39	10.66 ± 0.80
RC-160 plus DOX	1167 ± 34.14*	10.91 ± 0.93

DOX, doxorubicin.
*$P<0.05$ vs. all groups.
**$P<0.01$ vs. all groups.

High-performance liquid chromatography

Cytotoxic hormone analogues are hydrolyzed by CE in sera. We measured the changes in concentration of AN-162 in mouse and human sera by HPLC at various time intervals after adding 0.1 mg/ml of the compound and calculated the $t_{1/2}$. The $t_{1/2}$ of AN-162 in sera of nude mice was 17.1 ± 1.66 min. The $t_{1/2}$ of AN-162 in human sera was significantly longer, being 39.8 ± 2.3 min ($P < 0.001$) as compared with the $t_{1/2}$ in mice sera, which is because of a known higher activity of serum CE in mice sera. Additional kinetic studies of AN-162 were carried out in FBS ($t_{1/2} = 134.9 \pm 19$ min) and DMEM supplemented with 10% FBS as a typical percentage used for cell culture ($t_{1/2} = 157.5 \pm 3.6$ min) (Fig. 4).

Fig. 4



Hydrolysis of cytotoxic analog of somatostatin AN-162 after incubation at 37°C in a 95% air/5% CO_2 atmosphere with 100% humidity in Dubecco's modified essential media (DMEM) supplemented with 10% fetal bovine serum (FBS), of nude mice sera, human sera or FBS. AN-162 was quantified in the different sera using high-performance liquid chromatography as described in Materials and methods. $t_{1/2}$ were extrapolated.

Discussion

Peptides linked with chemotherapeutic agents, which can be targeted to hormone receptors on tumors are designed to produce a more selective and more effective delivery to malignant lesions, and therefore decrease the overall toxicity after repeated administration during the treatment. The direct delivery to malignant lesion also lowers the therapeutic dose of cytotoxic compounds compared with the cytotoxic moiety itself, which is part of the hybrid, thus contributing to the lower toxicity of the conjugate. In earlier studies, we showed LHRH-R mediated internalization of AN-152 [26] and showed that the growth of various human cancer cell lines xenografted into nude mice can be inhibited by this targeted cytotoxic analog. Similar results were obtained with targeted cytotoxic analog of SST AN-238 [11–18]. AN-152 consists of a LHRH analog linked with DOX through glutaric acid, whereas AN-238 contains SST analog RC-121 conjugated to 2-pyrrolino-DOX. 2-pyrrolino-DOX is a superactive derivate of DOX. Consequently, we decided to test targeted cytotoxic analog of SST AN-162 consisting of DOX conjugated through glutaric acid to SST octapeptide RC-121. The internalization and intracellular trafficking of the sstr–ligand complex was shown by many other groups using analogues or antagonists of SST. The degree of internalization is also receptor subtype selective [27–30]. We assume that once AN-162 binds to the receptors of SST the receptor-AN-162-complex will also be internalized and the cytotoxic moiety (DOX) is set free. As about 78% of human breast cancers are positive for sstr at protein levels (84% for sstr1, 79% for sstr2, 89% for sstr3, 68% for sstr4, 68% for sstr5) [31], we decided to use a human breast cancer cell line (MDA-MB-231) for our tests. We proved the expression of sstr in the MDA-MB-231 cell line with PCR. The binding of SST analog RC-160 to the membrane receptor protein of the cell line was shown by ligand competition assays. Our study shows for the first time that treatment with the targeted cytotoxic analog AN-162 inhibits growth of MDA-MB-231 human estrogen-independent breast cancer xenografts in nude mice. AN-162 produced a significantly greater inhibition of tumor growth than an equimolar dose of DOX. The tumors in mice treated with SST analog RC-160 or DOX showed no significant inhibition of tumor growth compared with the control group. This indicates that the tumor growth inhibition of AN-162 is likely mediated through the receptors of SST. The combination of RC-160 and DOX showed a small, but significant inhibition of tumor growth only at the end of the study on day 35. This seems to be because of an additive effect through the application of both substances. In contrast, the tumor doubling time showed no significant difference between the group of animals treated with the unconjugated mixture compared with the control group. There was no difference in the mean weights of animals treated with AN-162 and the control group during the whole study, which indicates that AN-162 is well tolerated. In the toxicity study, we found that the MTD for AN-162 in nude mice is 3.5 µmol/kg, whereas the MTD for SST analog RC-160 is 2.5 µmol/kg. The MTD for DOX was estimated to be 13.8–20.7 µmol/kg (8–12 mg/kg) [4,32]. These findings show that RC-160 is more toxic than the cytotoxic compound AN-162. As RC-121, the carrier for AN-162 is related to RC-160, the toxicity of this compound depends on the SST analog moiety. In the half-life experiments, we showed that the $t_{1/2}$ of AN-162 in nude mice sera is 17.1 ± 1.7 min compared with $t_{1/2}$ of 39.8 ± 2.3 min in human sera. We assume that the difference in the $t_{1/2}$ is because of a higher CE activity in mice sera than in human sera [19–21]. These results are supported by the findings of Nagy *et al.* [19] on half-life of AN-152 in which DOX is also linked with its carrier through glutaric acid. On account of the difference of the half-lives in sera of nude mice and human sera, the results from cancer xenograft models in nude mice have to be interpreted with caution with regard to translation into clinical studies. For clinical use, we expect a greater tolerance of AN-162 in humans with higher applicable doses because of the slower degradation in human sera than in mice sera. A slow i.v. infusion of AN-162 to avoid high peak levels in blood of the octapeptide carrier moiety might also permit the use of higher doses in humans to maximize the therapeutic effect. The results of the in-vivo toxicity study may contribute to a design of further experiments with the targeted cytotoxic SST analog AN-162. Collectively, our studies support the concept of targeted chemotherapy based on cytotoxic

peptide analogues for the treatment of breast cancers and other cancers expressing sstr.

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