

Potent in Vitro Antiproliferative Properties for a Triplatinum Cluster toward Triple Negative Breast Cancer Cells

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Abstract

The trinuclear platinum cluster $[Pt_3(\mu\text{-P}t\text{Bu}^t)_3(\text{CO})_3]\text{CF}_3\text{SO}_3$ (**I**) was designed featuring the presence of a nearly equilateral platinum triangle bridged by three di-tert-butylphosphide ligands; in addition, each platinum center bears a terminal carbonyl ligand. This triplatinum cluster was initially developed in view of applications in the field of cluster-containing innovative materials. Yet, due to the large success of platinum complexes in cancer treatment, we also decided to explore its cytotoxic and anticancer properties. Accordingly, the solubility profile of this compound in several solvents was preliminarily investigated, revealing a conspicuous solubility in DMSO and DMSO/buffer mixtures; this makes the biological testing of **I** amenable. UV-Vis measurements showed that the triplatinum cluster is stable for several hours under a variety of conditions, within aqueous environments. No measurable reactivity was observed for **I** toward two typical model proteins, i.e. Lysozyme and Cytochrome c. On the contrary, a significant reactivity was evidenced when reacting **I** with small sulfur-containing ligands. In particular, a pronounced reactivity with reduced glutathione and cysteine emerged from ESI-MS experiments, proving complete formation of **I**-GSH and **I**-Cys derivatives, with the loss of a single carbonyl ligand. Starting from these encouraging results, the cytotoxic potential of **I** was assayed in vitro against a panel of representative cancer cell lines, and potent cytotoxic properties were disclosed. Of particular interest is the finding that the triplatinum species manifests potent antiproliferative properties toward Triple Negative Breast Cancer Cells, often refractory to most anticancer drugs. Owing to the reported encouraging results, a more extensive biological and pharmacological evaluation of this Pt cluster is now warranted to better elucidate its mode of action.

Keywords: Pt clusters, cytotoxicity, Mass spectrometry, cancer

1. Introduction

Molecular platinum clusters have been intensely investigated during the last three decades for their attractive and peculiar chemical and electronic properties [1-4]. A variety of Pt clusters were thus prepared and characterised mostly containing a number of Pt atoms ranging from 3 to 50 [5-8]. In spite of these intense research activities and of extensive literature reports, we realised that this kind of compounds were poorly considered for their biological actions. This situation probably arises from the fact that Pt clusters are usually insoluble in aqueous environments and thus scarcely amenable for biomedical applications. In addition, their main application has been, traditionally, as models of heterogeneous platinum catalysts [9-11].

Yet, as platinum drugs play a key role in the current chemotherapeutic treatments of several cancers and as thousands of Pt analogues have been prepared and tested so far for this purpose, we wanted to ascertain whether selected Pt clusters might be of interest as prospective anticancer agents. The triplatinum cluster $[\text{Pt}_3(\mu\text{-P}(\text{t}\text{-Bu})_2)_3(\text{CO})_3]\text{CF}_3\text{SO}_3$, (**I**), prepared and characterised a few years ago in our laboratory [12], appeared to be a good candidate for this kind of investigations. Its crystal structure had been solved earlier, and a model of the cation is shown in Figure 1. This complex consists of three equidistant platinum centers connected through bridging P donors belonging to three di-*tert*-butylphosphide ligands; in turn, each platinum center bears a carbonyl ligand.

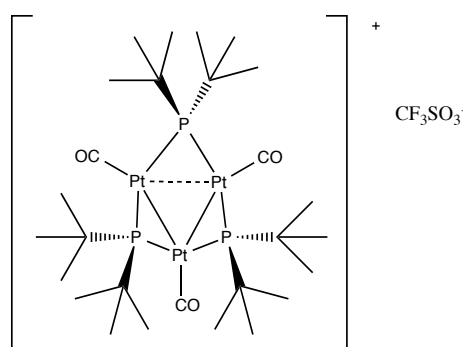


Figure 1. Structure of complex **I**.

This monocationic triplatinum cluster was first developed in view of its use as a building block for the synthesis of cluster-containing molecular assemblies [13]. However, as it also manifests an appreciable stability in solution and sufficient solubility even in aqueous environments, we wondered whether it might be suitable for biomedical applications.

These arguments led us to study in more detail the solution behaviour of **I** and to characterise its reactivity toward a few standard biomolecules. Afterward, the antiproliferative and pro-apoptotic properties of **I** were measured *in vitro* against a small panel of cancer cell lines with rather encouraging results.

2. Materials and Methods

2.1. Spectrophotometric Experiments

UV–vis absorption spectra were recorded on a Varian Cary 50 UV-Vis spectrophotometer (Varian, Palo Alto, CA, USA) in the range of 200–700 nm. Stock solution of **I** (3×10^{-3} M) was prepared by dissolving the complex under investigation in DMSO.

For stability studies in solution, UV-Vis measurements were performed by diluting the compound's stock solutions to 3×10^{-4} M in DMSO. Spectra were collected during 24 and 72 h at room temperature, operating in 10 min intervals during the first hour and in 1 h intervals afterward.

2.2. GSH and Cys Interaction Studies – Electrospray Ionization Mass Spectrometry.

The stock solution of **I** was prepared in DMSO at 1 mM and stored at -20 °C until usage. DMSO stock solutions (10 mM) of GSH and Cys were prepared. 100 µL of these solutions were added, separately, to 10 µL of the stock solution of **I** in order to obtain a 10:1 ratio. The mixtures were diluted with DMSO at a final concentration of 10^{-4} M of **I**. Samples were incubated at 37 °C for 24.

Electrospray ionization mass spectra of the mixtures were recorded from samples generated through 20-fold dilution with H₂O/CH₃CN 1:1 in an LTQ-Orbitrap high-resolution mass spectrometer (Thermo, San Jose, CA, USA), equipped with a conventional ESI source (direct introduction, flow rate 5 µL/min). The following standardized working conditions were applied: spray voltage 3.1 kV, tube lens voltage 230 V, capillary voltage 45 V, and capillary temperature 220 °C. Sheath and auxiliary gases were set at 17 au and 1 au, respectively. Data was acquired with a nominal resolution of 100 000 (at *m/z* 400), using Xcalibur 2.0 software (Thermo).

2.3. Biological Experiments

Cell cultures. MDAMB231, MCF7 cell lines were cultured in DMEM (Euroclone; Milan, Italy) with 10% Fetal Bovine Serum (FBS) (Euroclone Defined; Euroclone; Milan, Italy). HCT-

116, FLG 29.1, L929 and HL60 were cultured in RPMI 1640 (Euroclone; Milan, Italy) with 10% Fetal Bovine Serum (FBS) (Euroclone Defined; Euroclone; Milan, Italy). HCT116 cells were kindly provided by Dr R. Falcioni (Regina Elena Cancer Institute, Roma). We cultured the cell lines at 37 °C under a humidified atmosphere in 5% CO₂ in air.

Pharmacology experiments. Cells were seeded in a 96-well flat-bottomed plate (Corning-Costar, Corning, NY, USA) at a cell density of 1×10^4 cells *per* well in either RPMI or DMEM complete medium. The compound **I** was used, after solubilisation in water without DMSO, in range of concentration of 0-200 nM. After 24 h and 48 h, viable cells (determined by Trypan blue exclusion) were counted in triplicate using a haemocytometer. Each experimental point represents the mean of a single experiment carried out in triplicate.

Trypan blue assay. Cells viability was assessed by the Trypan blue exclusion assay. In brief, 10 µL of 0.4% trypan blue solution was added to 10 µL cell suspensions in culture medium. The suspension was gently mixed and transferred to a haemocytometer. Viable and dead cells were identified and counted under a light microscope. Blue cells failing to exclude the dyes were considered nonviable, and transparent cells were considered viable. The percentage of viable cells was calculated on the basis of the total number of cells (viable plus nonviable).

The IC₅₀ value (*i.e.*, the dose that caused apoptosis of 50% of cells) was calculated by fitting the data points (after 24 h and 48 h of incubation) with a sigmoidal curve using Calcsyn software (Biosoft, Cambridge, UK).

3. Results and Discussion

3.1. Chemistry: Solution Behaviour and Reactivity

The solubility profile of complex **I** was investigated in a variety of solvents. Beyond being soluble in some organic solvents like DCM and acetone, this compound also manifested an appreciable solubility in DMSO and, also, in DMSO/aqueous buffer mixtures.

The stability behaviour of the triplatinum cluster in these latter solvents was monitored over time by UV visible spectroscopy. As shown in Figure 2, this compound exhibits a typical absorption spectrum in the UV-Visible, characterised by the presence of four resolved bands at 280, 310, 330 and 380 nm. The triplatinum species was found to be stable during the first 12 hours at 25 °C.

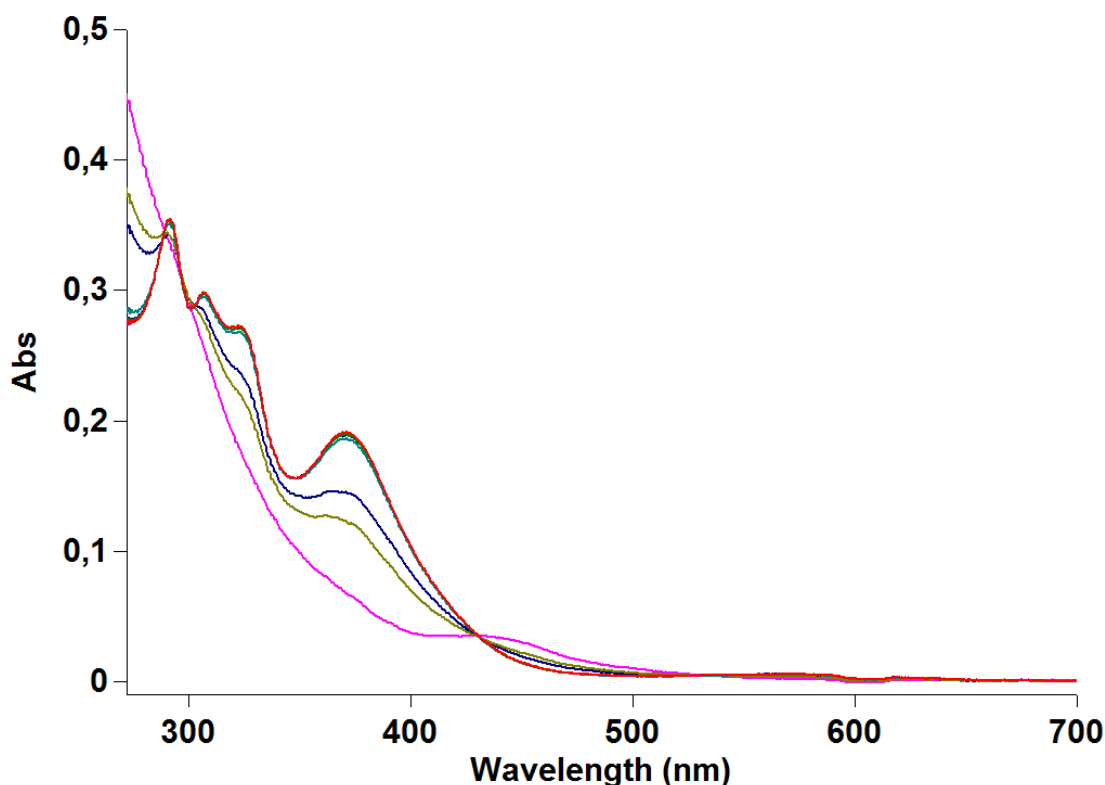


Figure 2. UV-Vis absorption spectra of a 3×10^{-4} M solution of **I** in DMSO recorded during 72h. The group of traces included with the red line are referred to the first 12h; blue line, 24h; green line, 48h; pink line, 72h.

Anyway, upon extending the stability study in DMSO up to 72 h at 25 °C, some major changes in the UV-Vis spectrum became evident (see Figure 2). In fact, a decrease of the bands located at 310, 330 and 380 nm is detected with a concomitant growing up of the bands at 250 and 440 nm. In addition, an isosbestic point is present, in the visible, at 420 nm.

Afterward, we analysed the reactivity of **I** with small proteins and a few low molecular weight biomolecules. ESI-MS turned out to be an optimal tool for this kind of investigation according to previous experience of our laboratory [14-16]. The ESI-MS spectrum of **I**, in CH_3CN solution, reveals the characteristic isotopic pattern (see Figure 3). No adduct formation was detected by ESI-MS when **I** was challenged against the two model proteins *cyt-c* and *lysozyme*, even after long incubation times. Yet, when reacted with small sulfur-containing ligands, a clear reactivity was highlighted. In particular, quantitative formation of stable derivatives with GSH and Cys was demonstrated by the ESI-MS experiments shown in Figure 4.

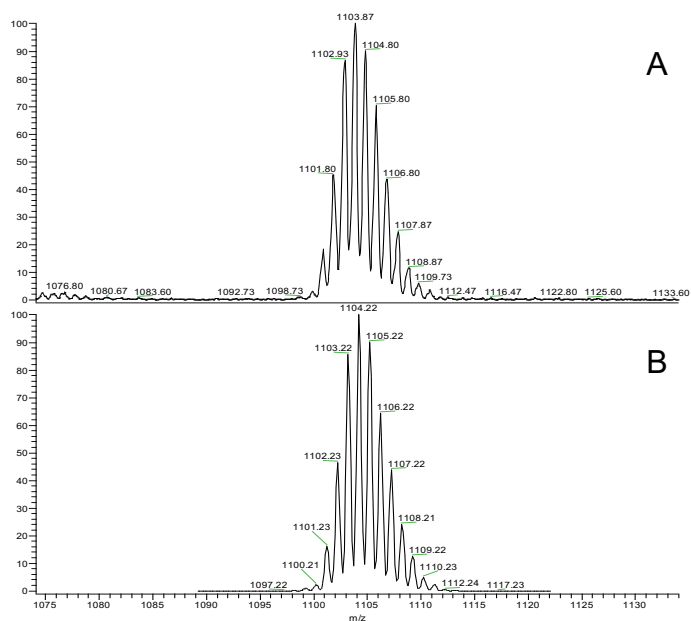


Figure 3. ESI-MS spectrum of **I**; panel A – measured, panel B – theoretical.

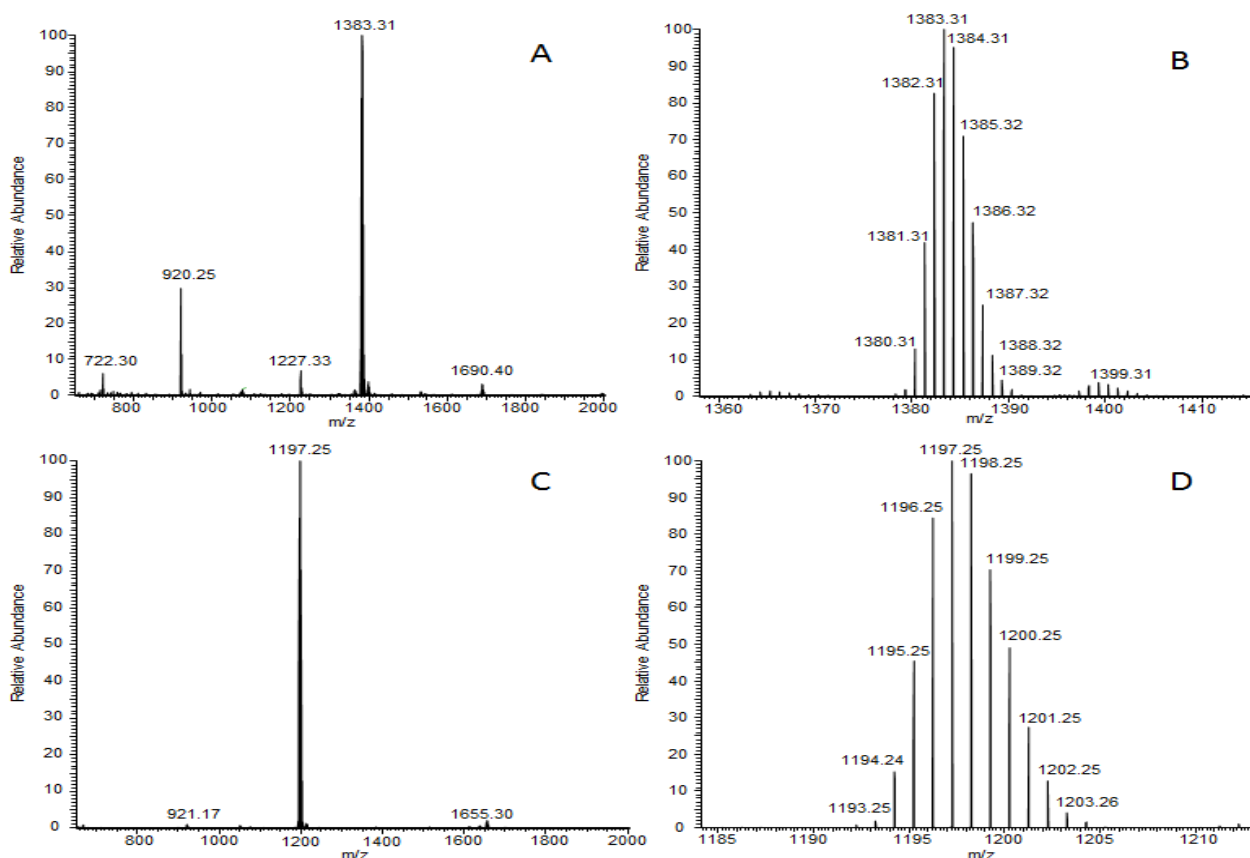


Figure 4. ESI-MS spectra of **I** in DMSO incubated with GSH (A) and Cys (B) with their characteristic isotopic patterns (B and D, respectively).

Indeed, from inspection of the ESI-MS spectra shown in Figure 4, the high reactivity of **I** toward both sulfur-containing small molecules is unambiguously documented. In fact, the peak at

m/z 1383.31 (panel A) could be assigned to the I-GSH complex, with the loss of only one CO group. In the same manner, the peak at m/z 1197.25 (panel C) was attributed to the I-Cys adduct. Even in this case the loss of one CO group occurs that is most probably displaced by a sulfur donor. Analysis of the measured isotopic pattern confirmed the formation of the two mentioned adducts (panel B and D, respectively). In addition, either in case of GSH or of Cys, the ESI-MS experiments did not reveal the presence of unreacted I nor the formation of adducts of I with more than one molecule of GSH or Cys.

3.2. Biological Studies

Having documented such an interesting behaviour of complex I in aqueous solution and its selectivity in biomolecular reactions, we decided to evaluate the cellular effects of complex I against a representative panel of cancer cell lines. The cancer cell panel included the following cell lines: MDAMB231 and MCF7 (human breast cancer), HL60 and FLG 29.1 (human acute myeloid leukemia), and a series of cell lines (human colorectal adenocarcinoma) showing different sensitivity to cisplatin *i.e.* HCT116, HCT8 and HT29.

To determine the cytotoxic effect of the triplatinum cluster on the above cancer lines we used the Trypan blue method as detailed in the experimental section. Following a preliminary appreciation of its potent antiproliferative effects, the biological activity of compound I was assessed at seven different concentrations in the nanomolar range, (*i.e.* 0.5 nM, 1 nM, 5 nM, 10 nM, 20 nM, 50 nM, 100 nM) after 24 and 48 hours incubation. Analysis of the corresponding growth curves according to Chou Talalay algorithm allows determination of the respective IC₅₀ values, that are reported in Table 1. The extremely high antiproliferative potential of this compound is thus demonstrated. The IC₅₀ values are higher after 48 hours of treatment, suggesting that the tested cancer cells are able to reverse, *i.e.* repair, at least partially, the insult. As this behaviour differs from cisplatin for which IC₅₀ values at 48 h are generally smaller than those recorded at 24 h, these results offer some initial evidence that the mode of action of the triplatinum cluster markedly differs from cisplatin.

Moreover, the cytotoxic activity against non-cancerous cell line L929 (mouse fibroblast), was also assessed in the same conditions. Normal cells turned out to be less sensitive to compound I than cancer cells (IC₅₀ 167.3±8.67 nM). Tumour selectivity values, calculated as the IC₅₀ values for primary cultures divided by the IC₅₀ values for the cancer cells, are in the range between 19.25 (MCF7) and 3.68 (HCT116). These data clearly indicate that the experimental compound I possesses a moderate to pronounced selectivity for tumour cells. In the same experimental conditions three cancer cell lines, HCT116, HT29 and HCT8 were also tested that possess variable

sensitivity to cisplatin, respectively (see Table 1). The obtained data clearly showed that cell line less sensitive to cisplatin has the lower IC₅₀ for compound I.

Table 1. IC₅₀ values for compound I in a panel of leukaemia cell lines and solid tumour cell lines.

Cell line	Type	IC ₅₀ (nM) 24h	IC ₅₀ (nM) 48h	IC ₅₀ Cisplatin (μM)
MDAMB231	Breast cancer	16.3±0.8	19.6±1.1	82.5±3.2
MCF7	Breast cancer	8.7±1.1	32.1±1.2	-
HL60	Acute leukemia	22.5±0.8	37.8±1.4	-
FLG 29.1	Acute leukemia	31.6±0.9	69.5±2.4	24.4± 0.8
HCT116	Colorectal adenocarcinoma	45.4±6.5	61.2±3.7	25.5± 2.1
HT-29	Colorectal adenocarcinoma	130.0±14.5	-	16.6± 0.9
HCT-8	Colorectal adenocarcinoma	92.0±7.1	-	8.7±1.4

The cell lines were exposed to increasing concentrations of compound I in the range 0–200 nM. After 24 and 48 hours, viable cells (determined by Trypan blue exclusion) were counted in triplicate using a haemocytometer. Each experimental point represents the mean of four samples carried out in three separate experiments.

We next evaluated the effect of compound I in the induction of apoptosis in HCT116 cells by the Annexin/PI test. The treatment with compound I for 48 hours caused an evident induction of apoptosis as shown in Figure 5.

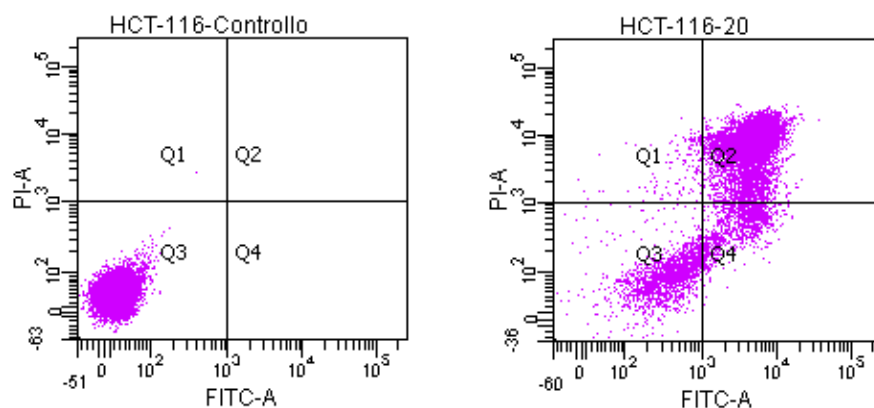


Figure 5. Effect of compound I on apoptosis induction of HCT116. Effect of compound I given at IC₅₀ value on HCT116 cells after 48 hours of treatment. Two representative panels are reported. Left panel = control (early apoptotic cells, Annexin+/PI-: 0%; late apoptotic cells, Annexin+/PI+: 0%); right panel = treated with compound I (early apoptotic cells, Annexin+/PI-: 12%; late apoptotic cells, Annexin+/PI+: 39%).

4. Conclusions

From these initial studies it can be stated that the investigated triplatinum cluster manifests an appreciable stability within various physiological media and exhibits, at the same time, very potent antiproliferative effects in a panel of representative tumor cell lines. It is remarkable that a very potent cytotoxic action is detected also toward the triple negative MDAMB231 cells, a kind of cancer cell line often refractory to several medical treatments. It will be of interest in the next future to perform further studies to better understand the mode of action of this novel and peculiar Pt complex. It is particularly relevant to determine whether the cluster acts in its intact form, *i.e.* as a trimetallic species, or undergoes degradation in the cellular environment releasing monometallic Pt species.

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Abbreviations

Cys cysteine

DCM dichloromethane

DMEM Dulbecco's Modified Eagle Medium

DMSO dimethyl sulfoxide

ESI-MS electrospray ionization mass spectrometry

GSH reduced glutathione

HER2 human epidermal growth factor receptor 2

MCF 7 Michigan Cancer Foundation-7

PI propidium iodide

RPMI Roswell Park Memorial Institute medium

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