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Differences in protein binding and excretion of Triapine and its Fe(III) complex

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ABSTRACT

Triapine has been investigated as anticancer drug in multiple clinical phase I/II trials. Although promising anti-leukemic activity was observed, Triapine was ineffective against solid tumors. The reasons are currently widely unknown. The biological activity of Triapine is strongly connected to its iron complex (Fe-Triapine) which is pharmacologically not investigated. Here, novel analytical tools for Triapine and Fe-Triapine were developed and applied for cell extracts and body fluids of treated mice. Triapine and its iron complex showed a completely different behavior: for Triapine, low protein binding was observed in contrast to fast protein adduct formation of Fe-Triapine. Notably, both drugs were rapidly cleared from the body (serum half-life time <1 h). Remarkably, in contrast to Triapine, where (in accordance to clinical data) basically no renal excretion was found, the iron complex was effectively excreted via urine. Moreover, no Fe-Triapine was detected in serum or cytosolic extracts after Triapine treatment. Taken together, our study will help to further understand the biological behavior of Triapine and its Fe-complex and allow the development of novel thiosemicarbazones with pronounced activity against solid tumor types.

1. Introduction

Due to their high proliferation rate, tumor cells are characterized by sensitivity to iron deprivation. With the aim of targeting this iron dependency, several iron chelators have been developed for cancer treatment [1]. Among them, α-N-heterocyclic thiosemicarbazones belong to the most promising compound class with Triapine (3-aminopyridine-2-carboxaldehyde thiosemicarbazone; Fig. 1A) as the most promising representative [2]. Thiosemicarbazones are able to efficiently inhibit the enzyme ribonucleotide reductase (RR), which is strongly dependent on the intracellular iron pools [3]. Disruption or inhibition of RR leads to dNTP pool depletion and, consequently, blocks DNA synthesis and cell cycle progression. As cellular iron is utilized in multiple homeostatic processes, also other iron-dependent pathways are affected by thiosemicarbazone treatment [4]. Furthermore, in some cases, e.g. for the Triapine derivative Dp44mT (di-2-pyridylketone-4,4,-dimethyl-3-thiosemicarbazone), in addition also other metal ion pools like copper are targeted [1].

It is widely accepted that RR inhibition by Triapine is based on the interaction with the diiron cofactor-containing subunit R2 of the enzyme, which is essential for enzymatic activity. Central in this proposed mode of action of Triapine is its iron complex (Fe-Triapine, Fig. 1A), which is supposed to destroy the tyrosyl radical of the R2 subunit probably by direct interaction [5]. In addition to RR, recently also other targets like endoplasmatic reticulum stress induction have been suggested for Triapine [6]. Notably, the direct proof of Fe-Triapine formation in the body is still missing [7] and so far basically no in vivo data are available regarding pharmacokinetics or —dynamics of the proposed iron complex.

With regard to the clinical practice, Triapine showed promising activity against advanced leukemia [8–11]. However, although it is active in xenograft models [12,13], clinical phase I/II studies revealed that

Abbreviations: 5-HP, 5-hydroxypicolinaldehyde thiosemicarbazone; DMSO, dimethyl sulfoxide; dNTP, deoxynucleoside triphosphate; Dp44mT, di-2-pyridylketone-4,4,-dimethyl-3-thiosemicarbazone; EIC, extracted ion chromatogram; EPR, electron paramagnetic resonance; FCS, fetal calf serum; Fe-Triapine, iron(III) complex of Triapine; FLD, fluorescence detection; HPLC, high performance liquid chromatography; ICP-MS, inductively coupled plasma mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; MS, mass spectrometry; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered saline; RR, ribonucleotide reductase.

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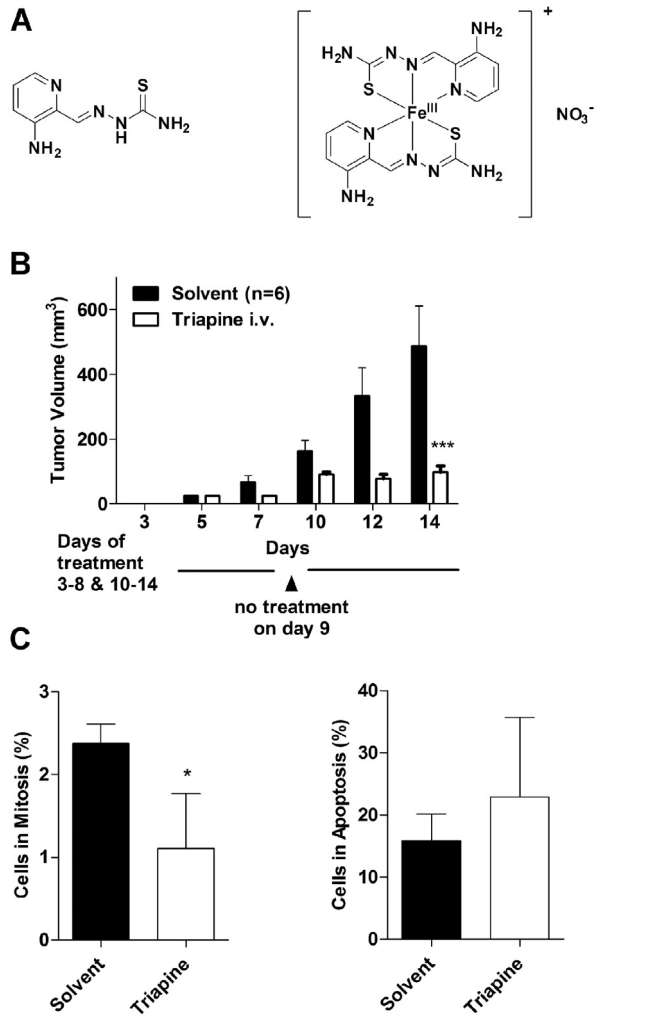

Fig. 1. A) Molecular structures of Triapine and Fe-Triapine. B) Anticancer activity of Triapine in vivo. Murine CT-26 xenografts were grown in Balb/c mice and treated with Triapine·HCl (5 mg kg^{-1}) intravenously. Days of therapy are indicated by a black bar. Treatment break at day 9 is indicated by an arrow. At the last day of treatment tumor samples were collected, paraffin-embedded and slices prepared. C) Percentages of mitotic and apoptotic cells were evaluated microscopically by counting H/E-stained tumor dissection (n = 3 from n = 4 mice). For statistical analyses 2-way ANOVA with Bonferroni post-correction was performed (*$p < 0.05$, ***$p < 0.001$) using Graph Pad Prism software.

Triapine mono-treatment is widely ineffective against several solid tumors including advanced pancreas adenocarcinoma [14], non-small-cell lung cancer [15], or renal cell carcinoma [16]. The reasons for this are currently unknown and might be based on the inappropriate drug delivery into the solid tumor nodules (the plasma half-life time of Triapine is only ~1 h [17,18]), fast excretion/metabolism and/or intrinsic/acquired drug resistance. Therefore, it is of high interest to gain more insights into the pharmacological characteristics of Triapine, especially in comparison to its supposed active species, the sparsely investigated iron complex (Fe-Triapine). Thus, in this study analytical tools were developed and used to trace the fate of Triapine as well as its iron(III) complex in several biological fluids. By this, it was shown that Triapine is characterized by a high drug stability and low protein binding affinity. In contrast, Fe-Triapine rapidly binds to proteins in cell culture and in vivo. Interestingly, electron paramagnetic resonance (EPR) measurements revealed no significant levels of Fe-Triapine in cell culture or in serum of mice after treatment with Triapine, which sheds new light on the role of Fe-Triapine in the activity of Triapine.

2. Materials and methods

2.1. Analytical materials

Triapine (3-aminopyridine-2-carboxaldehyde thiosemicarbazone), Triapine·HCl and the iron(III) complex Fe-Triapine ([bis (3-aminopyridine-2-carboxaldehyde thiosemicarbazonato)-N,N,S-iron(III)] nitrate) (Fig. 1A) were synthesized according to literature procedures [19]. Formic acid (98–100%, Merck, Germany), ammonium formate (>99%, Fluka, Austria) and sodium chloride (99.99%, Merck) were of Suprapur® quality. Acetonitrile and water (Fluka) were of LC–MS grade. Proteins as well as all other chemicals were obtained from Sigma Aldrich, Austria. Fluorescence measurements were performed on a Horiba FluoroMax®-4 spectrofluorometer. Scans were run at room temperature with excitation and emission slit widths of 4 nm. Emission scans were run between 400 and 600 nm (excitation wavelength 300–400 nm).

2.2. Chromatographic separation

The chromatographic separation of Triapine and Fe-Triapine was performed with an Atlantis T3 C18 reversed-phase column (150 mm × 2.1 mm, 3 μm particle size) from Waters (Milford, USA). As a mobile phase a gradient prepared from an aqueous 5 mM solution of ammonium formate adjusted to pH 6 with formic acid (eluent A) and acetonitrile containing 1% (v/v) water and 0.1% (v/v) formic acid (eluent B) was used. The mobile phase was kept constant at 10% B for 1 min. Then, B was increased to 50% within 5 min and kept for 2 min. Subsequently, B was increased to 90% within 0.1 min and kept for 0.9 min to flush the column, followed by reconstitution of the starting conditions within 0.1 min and re-equilibration with 10% B for 4.9 min (total analysis time = 14 min).

2.3. HPLC-FLD and HPLC–MS system

Due to the high affinity of Triapine for metal ions even within a HPLC system, an inert HPLC system was used. First attempts using high performance liquid chromatography mass spectrometry (HPLC–MS) showed poor ionization and, therefore, a high limit of detection (LOD)/limit of quantification (LOQ) which made it unsuitable for the quantification of low Triapine concentrations in biological samples. Thus, the determination of Triapine was carried out on an inert HPLC system connected to a fluorescence detector (HPLC-FLD), as the ligand shows intrinsic fluorescence properties (λ_{em} = 457 nm), upon excitation at λ_{ex} = 360 nm [20]. In particular, an Ultimate 3000 Standard HPLC System, Dionex, USA with a fluorescence detector was used and processed using Chromeleon software (Version 6.7, Dionex, USA).

The determination of the respective iron(III) complex Fe-Triapine was performed via MS-detection due to its positive charge and, therefore, low LOD/LOQ. The analysis was carried out on a HPLC coupled to an electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS) system (6210 TOF, Agilent Technologies, USA), utilizing a dual ESI interface in positive ionization mode. The following optimized parameters were used: drying gas 10 L min^{-1} (350 °C), nebulizer pressure 35 psi, capillary voltage 3000 V, fragmentor voltage 180 V and skimmer voltage 80 V. The mass spectrometer was connected to a HPLC system (1100/1200 Agilent Technologies). Mass Hunter software B.02.00 was used for instruments control and data evaluation.

For both detection methods (HPLC-FLD and HPLC–MS) flow rate 0.2 mL min^{-1}, injection volume 5 μL, column temperature 25 °C and autosampler temperature 5 °C was used.

2.4. Sample preparation for analytical measurements

To determine the content of free drug in biological samples, first the proteins were precipitated using acetonitrile. Subsequently, filtration in

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