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Electron Paramagnetic Resonance Study of Peripheral Blood Mononuclear Cells from Patients with Refractory Solid Tumors Treated with Triapine®

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Abstract

The metal chelator Triapine®, 3-aminopyridine-2-carboxaldehyde thiosemicarbazone, is a potent inhibitor of ribonucleotide reductase. EPR spectra consistent with signals from Fe-transferrin, heme, and low-spin iron or cupric ion were observed in peripheral blood mononuclear cells (PBMCs) obtained from patients treated with Triapine®. One signal that is unequivocally identified is the signal for Fe-transferrin. It is hypothesized that Fe uptake is blocked by reactive oxygen species generated by FeT₂ or CuT that damage transferrin or transferrin receptor. A potential source for the increase in the heme signal is cytochrome c released from the mitochondria. These results provide valuable insight into the *in vivo* mechanism of action of Triapine®.

Keywords

Triapine®, 3-aminopyridine-2-carboxaldehyde thiosemicarbazone; peripheral blood mononuclear cells (PBMCs); electron paramagnetic resonance (EPR); reactive oxygen species (ROS); iron; transferrin; transferrin receptor; ribonucleotide reductase; patients with solid tumors

1. Introduction

Triapine® (3-aminopyridine-2-carboxaldehyde thiosemicarbazone, 3-AP [Fig. 1]), a metal chelator, is a potent small molecule inhibitor of ribonucleotide reductase (RR), an enzyme that is important for cell division and tumor growth [1]. The RR enzyme is responsible for reducing the four ribonucleotides to their corresponding deoxyribonucleotides (dNTPs) required for DNA synthesis and repair. Human RR is composed of two subunits: hRRM1, which contains

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the nucleotide binding site, and hRRM2, which contains the metal binding site (Fig. 1) [2]. The hRRM2 subunit has a non-heme iron and a tyrosine free radical, which are required for the enzymatic reduction of ribonucleotides [2]. The RR enzyme contains two ferric ions coordinated by four carboxylates and two histidine ligands that are antiferromagnetically coupled by a μ -oxo bridge, making the metal site silent to EPR. Inhibitors of the hRRM2 subunit act by destroying the free radical. The reaction of the iron center with oxygen generates a protein-bound tyrosyl radical that is detectable by EPR. Recent evidence has shown that RR spontaneously loses the iron atoms [3], rendering it chelatable by small hydrophobic chelators such as Triapine[®]. Inhibition of RR disrupts DNA synthesis and repair, leading to apoptotic cell death [4].

EPR can be used to measure Triapine[®] effects in the cell. The tyrosyl radical is directly proportional to the enzymatic activity of RR. Triapine[®]-mediated inhibition of RR is predicted to result in a decrease in the tyrosyl radical and an increase in the Cu-Triapine[®] (CuT) and Fe-Triapine[®] (FeT₂) complexes. CuT and FeT₂ should form in cells and retain their structure or form an adduct, by analogy with previous studies with 2-formylpyridine thiosemicarbazone Cu(II), a precursor to CuT [5 and refs therein]. Short exposure of cells to monothiosemicarbazones has no deleterious effect on their survival, but iron and copper complexes inhibit cell proliferation in mice [6]. In addition, Triapine[®] inhibits Fe uptake from transferrin and induces the transferrin receptor at both the mRNA and protein levels [7]. Increases in Fe-transferrin complexes and the transferrin receptor are also expected [8,9]. Cytochrome c release occurs early in the apoptotic cascade [10], and increased cytochrome c release is anticipated following treatment with Triapine[®].

Recent evidence suggests that formation of the metal chelate of Triapine[®] and iron is essential to its cytotoxic effect [9]. Precursors to Triapine[®] sequester large quantities of iron from humans [11-13]. The Fe-Triapine[®] complex directly inhibits RR in a step that involves hydrogen peroxide [9], much as precursors to FeT₂ inhibit RR [14,15]. In addition, the Fe-Triapine[®] complex is redox active, forming reactive oxygen species (ROS) that can deplete intracellular glutathione and cause DNA strand breaks, similar to iron doxorubicin or iron bleomycin complexes [9].

Triapine[®] has anti-tumor effects, both *in vitro* and *in vivo*, and several clinical trials are currently being conducted to evaluate the safety and anti-neoplastic activity of Triapine[®] alone or in combination with other chemotherapeutics. In this study, the effect of Triapine[®] on EPR spectroscopy signals was evaluated in peripheral blood mononuclear cells (PBMCs) from patients with refractory solid tumors.

2. Experimental Design

2.1. Sample preparation

PBMCs were obtained by collecting 10 ml samples of whole blood into cell preparation tubes by standard methods. The tubes were immediately centrifuged and PBMCs were removed. Samples were collected prior to drug administration, and at 2, 4.5, and 22 h after the end of the Triapine[®] infusion. The dose of Triapine[®] was 60 mg/m². The cells were washed once with PBS. Draw time to freeze time was close to an hour. Cells were spun for 25 min, washed, spun again, and transferred to EPR tubes. A small aliquot of cells was removed for cell count and protein determinations by standard methods. The remainder of the PBMCs specimen (10⁷ cells in 0.3 ml) was placed into 4 mm O.D. quartz EPR tubes, frozen in liquid nitrogen, and then stored at -80°C. Human holo-transferrin (Calbiochem, EMD Biosciences, La Jolla, CA) was dissolved in 0.225 M mannitol, 0.75 M sucrose, and 20 mM hepes buffer (MSH, pH 7.5), and used as a standard (320 μ M).

2.2. EPR spectroscopy

For spectra at 77 K, quartz tubes filled with sample were placed in liquid nitrogen in a finger dewar and inserted into a standard TE₁₀₂ cavity and run on a Varian E109 Century Series spectrometer (Varian, Palo Alto, CA). For samples at 10 K, a Bruker E500 ELEXSYS spectrometer (Silberstreifen, Germany) with an Oxford Instruments ESR-9 helium flow cryostat (Tubney Woods, Abingdon, Oxfordshire, UK) and a Bruker DM0101 cavity (Silberstreifen, Germany) was used.

3. Results

3.1. EPR spectra of FeT₂ and CuT *in vitro*

EPR spectra were obtained for Triapine[®] upon the addition of cupric or ferric ion in DMSO and PBS (Fig. 2) to use as a template to recognize signals in PBMCs. A typical low-spin iron signal, FeT₂, with $g_z=2.196$, $g_y=2.138$, and $g_x=2.003$, and a cupric signal, CuT, with $g_{||}=2.191$ and $A_{||}=175$ G, were obtained. These EPR parameters compared favorably with those for the iron complex ($g_z=2.176$, $g_y=2.135$, and $g_x=1.998$, unpublished results) and copper complex ($g_{||}=2.204$, $A_{||}=186$ G) of 2-formylpyridine monothiosemicarbazone [16].

3.2. EPR spectra of PBMCs from patients before and after treatment with Triapine[®]

Spectra were obtained from PBMCs from three patients before treatment and at 2, 4.5, or 22 h after treatment with Triapine[®]. In the spectra for these patients, an increase of the Fe-transferrin signal at 2 h followed by a decrease was observed (Figs. 3-5). There were weak signals at $g'=4.3$ and $g=2$ in the spectrum for the sample before treatment (Fig. 3). The resolution of the three characteristic lines at $g'=4.3$ is adequate to assign this signal to Fe-transferrin [17]. Lines in the $g'=4.3$ region were observed at 1515, 1585, and 1625 Gauss and predicted to be at 1535, 1600, and 1620 Gauss [18]. At 2 h after treatment with Triapine[®], the signal at $g'=4.3$ increased, compared to the Fe-transferrin signal from the sample taken before administration of Triapine[®]. The concentration of the signal is about 15 μ M in 0.3 ml (4 nmole/10⁷ cells) as determined by comparison to the signal from human Fe-transferrin (Fig. 6). This Fe-transferrin signal—potentially from Fe-transferrin bound to transferrin receptor—decreased in spectra taken at 4.5 and 22 h. A strong $g=6$ signal was observed at 2 h, with a less intense signal at 4.5 h and little or no signal in the spectrum for PBMCs before or at 22 h after treatment with Triapine[®]. It is possible that the $g=6$ signal is a high-spin heme signal for cytochrome c, signaling apoptosis, but it is only certain that the signal is from heme without knowledge of which heme. There are weak signals in the $g=2$ region. The most prominent signal at $g=2$ was observed at 2 h (Fig. 5). The half-life of Triapine[®] in plasma is 5.47 $\pm$ 1.66 (SD) hours with a time to maximal (T_{max}) concentration of 0.10 $\pm$ 0.20 hours (unpublished preliminary results). The maximum concentration of Triapine[®], as measured at T_{max}, was 1.77 $\pm$ 0.43 μ g/mL. As such, the estimated plasma concentration 2 h after infusion was 1.45 μ g/mL. If 100% formed CuT, the concentration would be 7 μ M, at the limit of detection by EPR. Detection of low-spin iron complexes in the μ M range is more likely. The sharp signal most prominent at 4.5 h is assigned to a free radical signal for which the origin is not known.

In the set of spectra for Patient 2 (Fig. 4), the pretreatment spectrum is not shown because the sample was accidentally destroyed. Strong signals at $g'=4.3$ in the spectrum for PBMCs taken at 2 and 4.5 h after treatment with Triapine[®] are dominated by the characteristic signal from Fe-transferrin. In addition, there are signals at $g=6$ attributable to heme iron. The signal in the $g=2$ region for the PBMCs sample taken at 2 h is again suggestive of an FeT₂ or CuT aggregate. The sharp but weaker signal in the $g=2$ region, best seen at 2 or 22 h, is tentatively assigned to a free radical signal. The spectrum from PBMCs taken 22 h after treatment with Triapine[®] has weak signals at $g'=4.3$ and $g=2$. The signal at $g'=4.3$ is not resolved into the characteristic signal

for Fe-transferrin, so at best only a fraction of the signal could arise from Fe-transferrin, and some or all of the signal at $g'=4.3$ is from other non-heme high-spin iron sites.

In the spectra for Patient 3, weak signals at $g=2$ and $g'=4.3$ were observed (Fig. 5). The $g'=4.3$ signal probably arises from an Fe-transferrin signal, but the low signal-to-noise ratio makes definitive identification of this signal difficult. The signal at $g'=4.3$ from the spectrum for PBMCs taken from a patient 2 h after treatment with Triapine[®] has the characteristic signal from Fe-transferrin [17,18]. This signal intensity decreased in this patient at 4.5 and 22 h. The signals at $g=2$ are not identified because there are too many possibilities. The striking feature in the $g=2$ region is that there is a substantial increase at 2 h followed by a decrease at 4.5 and 22 h. Whether this signal is an aggregated form of FeT₂ or CuT or another low-spin iron or cupric signal is not known. Because the lines in the $g=2$ region for the pretreatment spectra from Patients 2 (Fig. 4) and 3 (Fig. 5) look similar, the origin of this signal is possibly from PBMCs as a result of treatment with Triapine[®]. The g -value for this line is 2.053, suggestive of a cupric site. Nevertheless, signals from FeT₂ or CuT cannot be ruled out, even though the signal is not the same as the signals in Fig. 2.

A signal from a commercial source of human Fe-transferrin was used to determine the concentration of Fe-transferrin in the spectra (bottom spectrum, Fig. 6). The peak to peak signal height was reduced to represent 15.2 μ M Fe-transferrin. The concentration of Fe-transferrin compared to the standard Fe-transferrin signal is 14.5 μ M or 1 nm per 10⁷ cells in 0.3 ml for the signal in the spectrum for Patient 1 at 2 h (Fig. 3); 14.2 μ M and 13.8 μ M for the signal for Patient 2 at 2 and 4.5 h, respectively (Fig. 4); and 8.1 μ M for the signal for Patient 3 at 2 h (Fig. 5). Residual spectra after subtraction of the Fe-transferrin signal are shown in Fig. 6. There is a non-heme Fe signal after subtraction of the signal for Fe-transferrin in the spectrum for Patient 2 at 2 h. This accounts for the poor resolution of the Fe-transferrin signal in this spectrum.

4. Discussion

Ribonucleotide reductases play a vital role in DNA synthesis by catalyzing the conversion of nucleotides to deoxynucleotides. DNA synthesis is inhibited without a balanced supply of deoxyribonucleotides. Anticancer agents that are good chelators for iron and have redox activity have been sought for the purpose of inhibiting ribonucleotide reductase by removing iron from the enzyme [19]. Whether these are complexes of iron and copper Triapine[®] or subsequent complexes formed as a result of the presence of Triapine[®] is difficult to determine from the EPR signals due to formation of adducts and aggregates of the metal complexes in complex biological samples. Because T is a tridentate ligand, CuT has an open coordination site, which forms adducts with sulfur from thiols and nitrogen donor atoms [5]. Triapine[®] is a potent inhibitor of ribonucleotide reductase [1 and refs. therein], but it is not certain if the inhibition is a result of removing iron from RR or if Triapine[®] forms a metal complex, which inhibits the activity of RR, or both.

In earlier studies with a precursor of Triapine[®], the preformed cupric complex of 2-formyl pyridine monothiosemicarbazone was a potent inhibitor of ribonucleotide reductase [20]. Triapine[®] is also a tridentate chelator that ligates Fe and other metals. Preformed Fe-Triapine[®] is a more potent inhibitor of ribonucleotide reductase than free Triapine[®] [21]. Earlier studies also showed that the iron complex of similar monothiosemicarbazones inhibits RR better than the free ligand [22,23]. It was proposed that Triapine[®] formed a complex with Fe(III), was reduced to Fe(II), generated reactive oxygen species, and quenched ribonucleotide reductase activity [1,7]. A similar scenario was reported for the cupric complex of 2-formylpyridine monothiosemicarbazone [24].

How iron and copper complexes and precursors of Triapine[®] decrease ribonucleotide reductase activity as well or better than metal-free Triapine[®] is a puzzling aspect of the monothiosemicarbazone drugs. The EPR signals about $g=2$ suggest that iron and copper metal complexes are formed. In addition, FeT₂ and CuT are redox active and generate ROS [1,7,19], perhaps accounting for the free radical signals at $g=2$.

One signal that is unequivocally identified is the signal for Fe-transferrin. It appears that Fe (III)-transferrin is not reduced. A likely explanation is that Fe uptake is blocked, possibly through generation of reactive oxygen species from FeT₂ or CuT that damage transferrin or transferrin receptor resulting in Fe-transferrin not being metabolized properly. In support of this idea, it is known that 2-formylpyridine monothiosemicarbazone Cu(II) inhibits cellular iron uptake as well as inhibiting ribonucleotide reductase [8]. Assuming that reactive oxygen species are generated by either FeT₂, CuT, or adducts of these complexes that would form either by replacing T or by occupying the open equatorial site of the tridentate cupric complex, other sites may also be damaged. The Fe-Triapine[®] complex has been shown in model systems to dramatically increase ROS [9].

Another site observed by EPR is heme iron. For one patient (Fig. 3), a greater than tenfold increase in heme iron was detected. One compelling hypothesis is that reactive oxygen species cause cell death and release of cytochrome c follows apoptosis. Upon release of cytochrome c, the heme is oxidized as measured by an increase in the heme signal. While this hypothesis is reasonable, the heme signal is an accumulation of all oxidized hemes, and more work is necessary to assign the increase in heme to cytochrome c. Nevertheless, administration of Triapine[®] is known to cause apoptosis [4].

In summary, this study is a novel first in human EPR evaluation of the effects of Triapine[®], identifying signals in the $g=2$ region, an Fe-transferrin signal, and potentially cytochrome c release from the mitochondria if the $g=6$ signal is from cytochrome c. Formation of metal complexes of Triapine[®] is likely essential to an anticancer effect, whether the mechanism is related to RR inhibition or the formation of ROS [1,7]. The signals observed primarily at 2 h are transient and disappear at 22 h. Nevertheless, if apoptosis is triggered, cell death follows. These results provide direct *in vivo* evidence for the increase in Fe-transferrin in PMBCs from patients receiving Triapine[®] and provide valuable insight into the *in vivo* mechanism of action of Triapine[®].

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Table of Abbreviations

PBMCs	peripheral blood mononuclear cells
RR	ribonucleotide reductase
dNTPS	deoxyribonucleotides
CuT	Cu-Triapine [®]
FeT₂	Fe-Triapine [®]
ROS	reactive oxygen species
Tmax	time to maximal

Fig. 1. Schematic for the structure of Triapine® and the di-iron binding site in ribonucleotide reductase [2].

Fig. 2.

EPR spectra of FeT₂ (0.67 mM) and CuT (0.5 mM) in 87% DMSO and 13% PBS. Spectrometer conditions: microwave freq. 9.187 GHz; incident microwave power 5 mW; modulation amplitude 5 G; modulation frequency 100 KHz; time constant 0.128 s; scan time 4 min; temp. 77 K; Varian E109 spectrometer.

Fig. 3.

EPR spectra of PBMCs from a patient before treatment (pre) with Triapine®, and 2, 4.5, and 22 h after treatment with Triapine®. Spectrometer conditions: microwave freq. 9.630 GHz; incident microwave power 5 mW; modulation amplitude 10 G; modulation frequency 100 kHz; time constant 0.082 s; scan time 83 s; 9 scans averaged; temp. 10 K; Bruker Eleksys spectrometer with Oxford cryostat.

Fig. 4. EPR spectra of PBMCs from a second patient 2, 4, and 22 h after treatment with Triapine®. Spectrometer conditions as in Fig. 3.

Fig. 5. EPR spectra of PBMCs from a third patient before treatment (pre) with Triapine[®], and 2, 4.5, and 22 h after treatment with Triapine[®]. Spectrometer conditions as in Fig. 3.

Fig. 6. EPR spectra after subtraction of the Fe-transferrin signal for Patient 1 at 2 h, Patient 2 at 2 and 4.5 h, and Patient 3 at 2 h. Bottom spectrum: gain adjusted to 15 μ M human Fe-transferrin in MSH, pH 7.4.