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MOLECULAR PHARMACOLOGY, 41:8-16

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ACCELERATED COMMUNICATION

In Vitro Evidence for Direct Complexation of ADR-529/ICRF-187 [(+)-1,2-bis-(3,5-Dioxo-piperazin-1-yl)propane] onto an Existing Ferric-Anthracycline Complex

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Received May 20, 1991; Accepted October 17, 1991

SUMMARY

ADR-529 protects against anthracycline cardiotoxicity, possibly by preventing free radical induction. We hypothesize that this occurs by ADR-529 forming a ternary anthracycline-iron-ADR-529 complex. This study used 200-MHz Fourier-transformed NMR to demonstrate the ability of ADR-529 to do this. Peak assignments were by proton-correlated spectroscopy and proton-carbon heteronuclear-correlated spectroscopy. Ga^{3+} served as a probe for Fe^{3+}, and D_2O was the system solvent. Doxorubicin and epirubicin were the studied drugs. Proton spectra of multiple combinations (including pure standards as controls) were obtained. Both Ga^{3+} plus ADR-529 and Ga^{3+} plus doxorubicin showed evidence of complexation, as seen by appropriate peak shifts and changes in the associated coupling constants. Ga^{3+} plus ADR-529 plus epirubicin showed complexation different from that of Ga^{3+} plus ADR-529 or Ga^{3+} plus doxorubicin and consistent with the proposed structure. We conclude that ADR-529 would be able to form a ternary complex with an existing anthracycline-Fe^{3+} complex in an isolated aqueous environment.

The anthracycline antibiotics are among the most useful and widely utilized anticancer drugs, with activity against a wide range of hematological and solid tumors. The most commonly used drugs in this class are daunorubicin, DOX, and EPI. These drugs have a substantial number of adverse effects, which limit the ability to increase the dose to a level at which a higher cure rate may be possible. These toxicities include myelosuppression, leading to an increased number of opportunistic infections and resulting in higher morbidity and mortality. However, this side effect can be readily treated with antibiotics (1) and other agents (2). A more difficult problem is the development of a chronic nonreversible cardiomyopathy that may cause congestive heart failure (3). This potential complication means that the total cumulative dose of anthracyclines is limited to preestablished limits. The challenge facing oncologists presently is to reduce substantially the chronic cardiomyopathy that may lead to congestive heart failure at cumulative doses of 550 mg/m^2 for DOX and 900 mg/m^2 for the less cardiotoxic analog EPI.

This study was supported by a grant from the National Health and Medical Research Council of Australia.

The mechanism of this cardiotoxicity has been studied extensively (for reviews, see Refs. 4–6) and is believed to be due to the formation of a number of free radical species, leading to the destruction of cardiac cells (7, 8).

In a recent clinical trial, the experimental cardioprotector ADR-529 (formerly referred to in the literature as ICRF-187) (see Fig. 4A) was clearly shown to reduce chronic cardiac toxicity greatly (9). Anthracycline cardioprotection has also been shown in a number of animal models (10–13). A hypothesis as to the mechanism of this cardioprotection was suggested (14). In essence, the anthracyclines have been shown to form a bidentate complex with Fe^{3+} in aqueous environments. This complex has been shown to be highly stable and produce the free radicals responsible for lipid peroxidation (15). Several alternate hypotheses have been suggested to explain the cardioprotective action of ADR-529. One suggests that intracellular glutathione, which is a ubiquitous intracellular compound, acts as the major intracellular reducing agent. It was suggested that it may act as a radical scavenger, and ADR-529 may work by increasing levels of glutathione. However, this hypothesis has been disproven (12). Further studies have shown that ADR-

ABBREVIATIONS: DOX, doxorubicin (Adriamycin); FT, Fourier-transformed; ^{1}H-COSY, proton-correlated spectroscopy; ^{1}H-^{13}C-HETCOR, proton-carbon heteronuclear-correlated spectroscopy; AMBER, assisted model building with energy refinement; MM2, molecular mechanics algorithm used for conformational analysis using energy minimization; EPI, epirubicin; ADR-529 (ICRF-187), (+)-1,2-bis-(3,5-dioxo-piperazin-1-yl)propane; EHPG, *N,N'*-ethylenebis(2-hydroxyphenylglycine); HBED, *N,N'*-bis(2-hydroxybenzyl)ethylenediamine-*N,N'*-diacetic acid.

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529 does not protect against free radical damage produced from another class of anticancer drugs, i.e., bleomycin (16), suggesting a structure-specific response. This study has sought to test the hypothesis that ADR-529 acts as a tetradentate ligand binding to the existing Fe^{3+}-anthracycline complex enclosing the iron moiety and thus sterically hindering the formation of the free radical species.

Experimental Procedures

Materials

The computer modeling was conducted on a Silicon Graphics 4D-70GT workstation (Silicon Graphics, Cupertino), using Insight II (version 1.1.0, last updated August 1990) (Biosym Technologies, San Diego, CA), Discover (version 2.6.0, last updated August 1990) (Biosym Technologies), and Sybil software (version 5.2, last updated February 1989) (Tripos Associates, St. Louis, MO), and on a MacIntosh IIx personal computer (Apple Computer, Cupertino), using Chem3D+ software (version 2.01, last updated August 1989) (Cambridge Scientific Computing, MA). The structural (physical) models of the drugs and their proposed complexes were made with Cochranes of Oxford molecular model kits, using the orbit system (Aldrich Chemical Co., Milwaukee, WI) and the Minit system (Sigma Chemical Co., St. Louis, MO). The FT-NMR was conducted on a Varian Gemini 200-MHz NMR spectrometer (Varian Associates, Sunnyvale). The drugs used in this study (EPI, DOX, and ADR-529) were provided by Farmitalia Carlo-Erba (Australia). The gallium salt was AR grade and purchased from Sigma; the D_2O was Spectrosol grade, with a specified minimum isotopic purity of 99.75%, and was purchased from BDH Chemicals Australia (Kilsyth, Australia). The NMR tubes were 5 mm in diameter (model 507-PP, specified for 200-MHz applications; Wilmad, NJ).

All chemical manipulations were performed within a laminar flow cytotoxic drug safety cabinet (Email; Electrical Engineering Division Air Handling Products, Regents Park), within a C1 certified laboratory, using appropriate safety gowns, gloves, high filtration face masks, and face shields under clean-room conditions.

Methods

Computational methods. The different programs used in this study used different algorithms and equations to determine the same parameters. Each of the various calculations (AMBER, MM2 minimization, electrostatic volumes, etc) was made using a module from one of these programs, and the result was exported for further work by the other programs. Each particular module was chosen because it offered a distinct advantage over the others (e.g., ease of use, speed of calculations, closer correlation with NMR and crystallographic data where available, etc).

The structures of the relevant molecules were sketched onto the screen and minimized using AMBER (17) and MM2 (18) energy minimization. These structures are in agreement with previously reported conformations of these agents (19). The proposed ADR-529-Fe^{3+} complex (Fig. 1A) was drawn and minimized using MM2 routines. This served to elucidate whether it was geometrically feasible for such a complex to exist. The proposed ADR-529-Fe^{3+}-anthracycline complex (Fig. 1B, shown with EPI as the representative anthracycline) was similarly constructed by adding the ADR-529-Fe^{3+} onto the minimized structure of EPI, followed by an additional MM2 minimization to give the final structure.

To get an indication of the electrostatic feasibility of the proposed complex formation, the electrostatics module of Sybil was used to produce various charged potential volumes for both the anthracyclines and ADR-529 (data not shown). The molecules were brought closer in increments, such that the energy potential was minimized, to get a most probable path of approach. All energies (electrostatic, Van Der Waals, hydrogen bonding, etc.) were summed, and the resultant field was displayed as a color-coded volume. Three representative planes

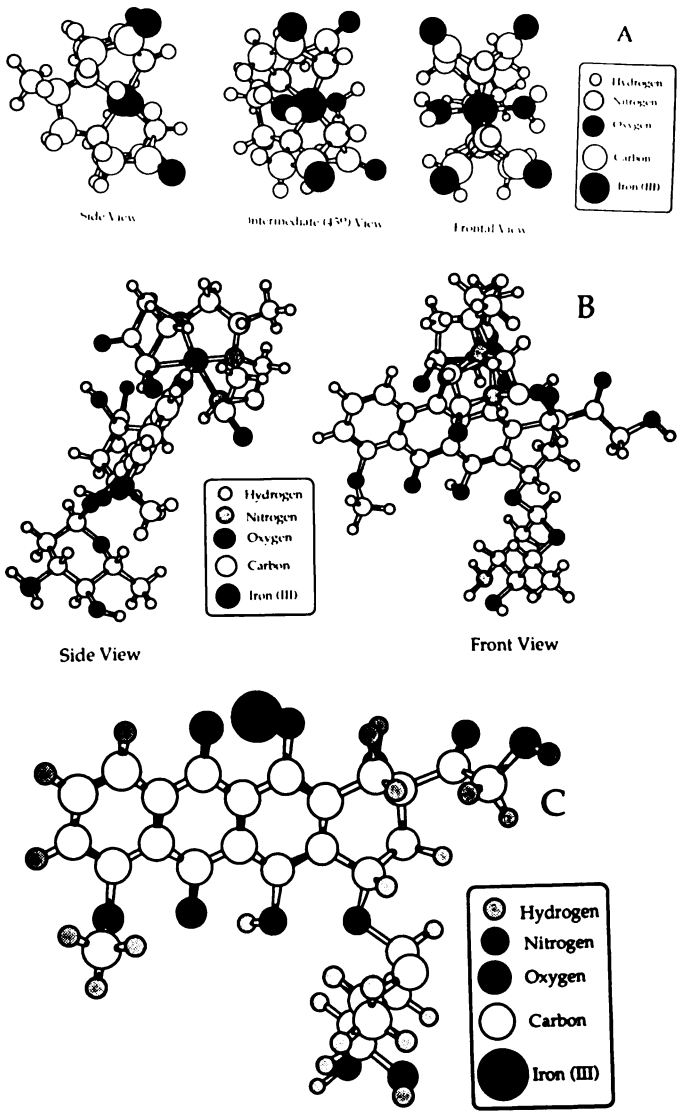

Fig. 1. Proposed ADR-529-Fe^{3+} complex (A), proposed ADR-529-Fe^{3+}-anthracycline complex (B), and the known Fe^{3+}-anthracycline complex (C).

were chosen for observation. Blue was used to represent an overall negative value (−1 kcal/mol), yellow for zero potential (0 kcal/mol), and red for positive potentials (+1 kcal/mol). The molecules were allowed to travel along the negative and zero potential planes and were repelled if they entered a positive node. The simulated approach served to qualitatively evaluate the possibility of complex formation as proposed.

Sample preparation. The concentration of anthracyclines used in this study was 36 mg/ml. The reason for this was to get the best possible signal to noise ratio in the smallest time possible, while remaining just under the critical level where extensive oligomerization occurs. Because this is 1000 times greater than the highest values seen in patients (20, 21), attempts were made to evaluate the same results at concentrations approaching clinical values. Samples were diluted 1/100 and 1/1000, and the spectra were observed. Observation times were substantially elongated and the obtained noise to signal ratios were very poor; however, the spectra at the reduced concentrations (data not shown) were superimposible over those obtained at higher concentrations, leading to the conclusion that it is plausible that these complexes may form and be stable at clinically relevant concentrations.

Twenty-five milligrams of DOX (molecular mass, 543.5 g/mol) or EPI (molecular mass, 543.5 g/mol) were placed in 10- × 75-mm boro-

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silicate glass culture tubes (Kimble Glass, Vineland), and 700 μl of D_2O were added with gentle agitation. The tubes were placed within amber polystyrene tubes on cotton wool plugs and tightly sealed with opaque white polypropylene caps. The assemblies were then agitated at room temperature for 5 min in reduced lighting and then covered with aluminium foil and stored at 4° for periods of 24–72 hr with 5-min agitations each 24-hr period until complete dissolution was obtained. The drugs dissolved to give deep burgundy- and deep orange-colored solutions, respectively. Samples were prepared in duplicate at several different times. No spectral differences were noted.

The samples were then transferred to NMR tubes and spectra were obtained. The samples were then stored with the NMR tubes placed on cotton wool plugs in amber polystyrene tubes with cotton wool wrapped around the upper perimeter of the amber tube above the level of the solution within the NMR tubes and kept at 4° without aluminium foil. The spectra of these samples taken several weeks later showed no observable differences.

Twenty-five milligrams of ADR-529 (molecular mass, 268.28 g/mol) were prepared in a similar manner as the anthracyclines (even though this compound is known to be insensitive to light), to ensure identical methods of preparation for all experiments. The resulting solution was clear and colorless, with no apparent change in viscosity.

Twenty-five milligrams of gallium(III) nitrate (molecular mass, 255.74 g/mol) were dissolved in a similar manner, to give a clear and colorless solution with no apparent change in viscosity.

Combinational experiments were performed by adding dry compounds to the borosilicate tubes, with mechanical mixing, followed by the addition of the D_2O solvent and dissolution as before.

All combinations were performed with 25 mg of each compound, resulting in 2:1 molar ratios for gallium and anthracyclines; 2:1 molar ratios for ADR-529 and anthracyclines; 1:1 molar ratios for gallium and ADR-529; and 2:1:1 molar ratios for ADR-529 and gallium and the anthracyclines.

No color changes were noted for the combination of ADR-529 and Ga^{3+}. There was a very slight discoloration with DOX and Ga^{3+}. No color changes were noted with DOX and ADR-529. A marked change in tone of color was noted for the ternary combination of DOX, Ga^{3+}, and ADR-529, to give a very deep magenta solution that was visibly increased in viscosity.

FT-NMR methodology. All NMR experiments were done at ambient temperature, using commonly employed pulse sequences. The ^1H NMR used a conventional pulse sequence with a frequency of 200 MHz, a spectral width of 3000.3 Hz, an acquisition time of 2.6 sec, a delay of 2.5 sec, and a pulse width of 17.0 μsec. The decoupler was tuned at the water frequency and power was adjusted to suppress the water signal produced by exchangable hydrogens. The ^1H-COSY pulse sequence had both spectral widths set to 930.1 Hz, frequency of 199.975 MHz, acquisition time of 0.998 sec, relaxation delay of 1.0 sec, pulse width of 45°, and first pulse of 90°, repeated 32 times in 128 increments. It was proton decoupled at 1300 db, with the decoupler gated off during acquisition. The ^1H-^{13}C-HETCOR sequence was set to observe carbon, with frequency of 50.289 MHz, one-dimensional spectral width (F2) of 2794.1 Hz, two-dimensional spectral width (F1) of 930.1 Hz, acquisition time of 91.6 msec, and pulse width of 90°, repeated 512 times in 64 increments. The decoupler was turned on at 20 db and broadband modulated.

Results

Computer Modeling and Simulation

The graphics modeling of the proposed complexes showed that the formation of the proposed structures was geometrically possible. Physical Orbit and Minit models also confirmed the structural feasibility of this complex.

Both molecules have negative potential energy nodes around the keto-hydroxy active site on the anthracene rings. In the simulation, the ADR-529 approached initially as a linear conformed molecule, and as it approached the Fe^{3+}-anthracycline complex a rotation about the central carbon backbone occurred under the prevailing mutual interaction of energy nodes, to form a cage-type conformation and displace the water ligands to complete the octahedral field around the complexed iron moiety. The resultant net total energy was lower than that for the hydrated complex. As the molecules approached each other, they mutually induced dipole moments on each other, the net effect of which was to accelerate the approach and further define the path of approach.

FT-NMR Spectroscopy

DOX (peak assignments). Structural assignments are shown on the COSY spectrum of DOX (Fig. 2A). In these experiments the proton spectrum appears along the diagonal, and projection onto either axis gives the spectrum. The peaks not on the diagonal (cross-peaks) appear only between coupled spin systems and form a square. This enabled assignment of the proton spins and subsequent skeleton tracing of the backbone of the molecule via cross-peak correlations. Coupling constants were taken by direct measurement of the separations of the peaks, expressed in Hz. Proton spectra (one-dimensional, normal) are shown on each axis. The assignment starts at the easily identified peak at 1.1 δ (the methyl doublet 27-CH_3) (22). The large square correlates the methyl (27-CH_3) to the hydrogen (26-H) at 4 δ and then to 25-H (3.5 δ), 24-H (4.6 δ), 23-H (1.6 δ), and the other easily assigned proton, 22-H at 5.1 δ. Two weak cross-peaks correspond to long-range, four-bond coupling (^4J) between 22-H and 24-H (4.6 δ) and to 18-H (3.5 δ). Hydrogen 18 then has a strong correlation to the nonequivalent 17-H, which then form their own square around 1.6 δ and 2.0 δ. This completes the connectivity plot (Fig. 2A). The only peaks remaining are H_2O, which is a broad singlet at 4.4 δ, and the aromatic hydrogens (Fig. 2B), which were assigned as 2-H at the lowest chemical shift (6.85 δ) adjacent to the -OCH_3, which is a broad doublet split by 2-H. 4-H is also a doublet at 6.95 δ split by 3-H, and 3-H is a triplet at 7.2 δ. The hydroxyl hydrogen atoms have been exchanged with deuterium from the solvent (D_2O), which gives a residual HDO peak at 4.6 δ. This solvent peak has been minimized in intensity by presaturation (decoupling). The triplet at 0.9 δ, together with the quartet at 3.5 δ and singlet at 2.1 δ, is consistent with a small amount of ethyl acetate being present as an impurity. The proton spectrum of EPI is not significantly different from that of DOX in the region where the formation of the complex is expected (data not shown) and, thus, within the context of these experiments the two drugs are interchangable.

Gallium. The iron(III) cation has a spin-unpaired electron and thus causes appreciable interference in the magnetic field of the NMR spectrometer. To overcome this problem, gallium(III) was used as a probe. Gallium is not generally regarded as being a biologically relevant ion nor is it a transition metal ion, therefore raising the issue of the selection of this species as an iron probe. Gallium(III) and iron(III) have the same charge and very similar ionic radii (0.62 Å and 0.64 Å, respectively) (23). This compares favorably with copper(II), whose ionic radius is 0.72 Å (23) and which is also known to complex with DOX (24) (the ionic radius of Fe^{2+} is 0.74 Å) (23). Thus, both size and charge are conserved. The affinities of Fe^{3+} and Ga^{3+} for biologically relevant ligands are similar. For transferrin, their respective log formation constants (logK_f) are 20.7 and 20.3 (25). With regards to other clinically relevant EDTA-

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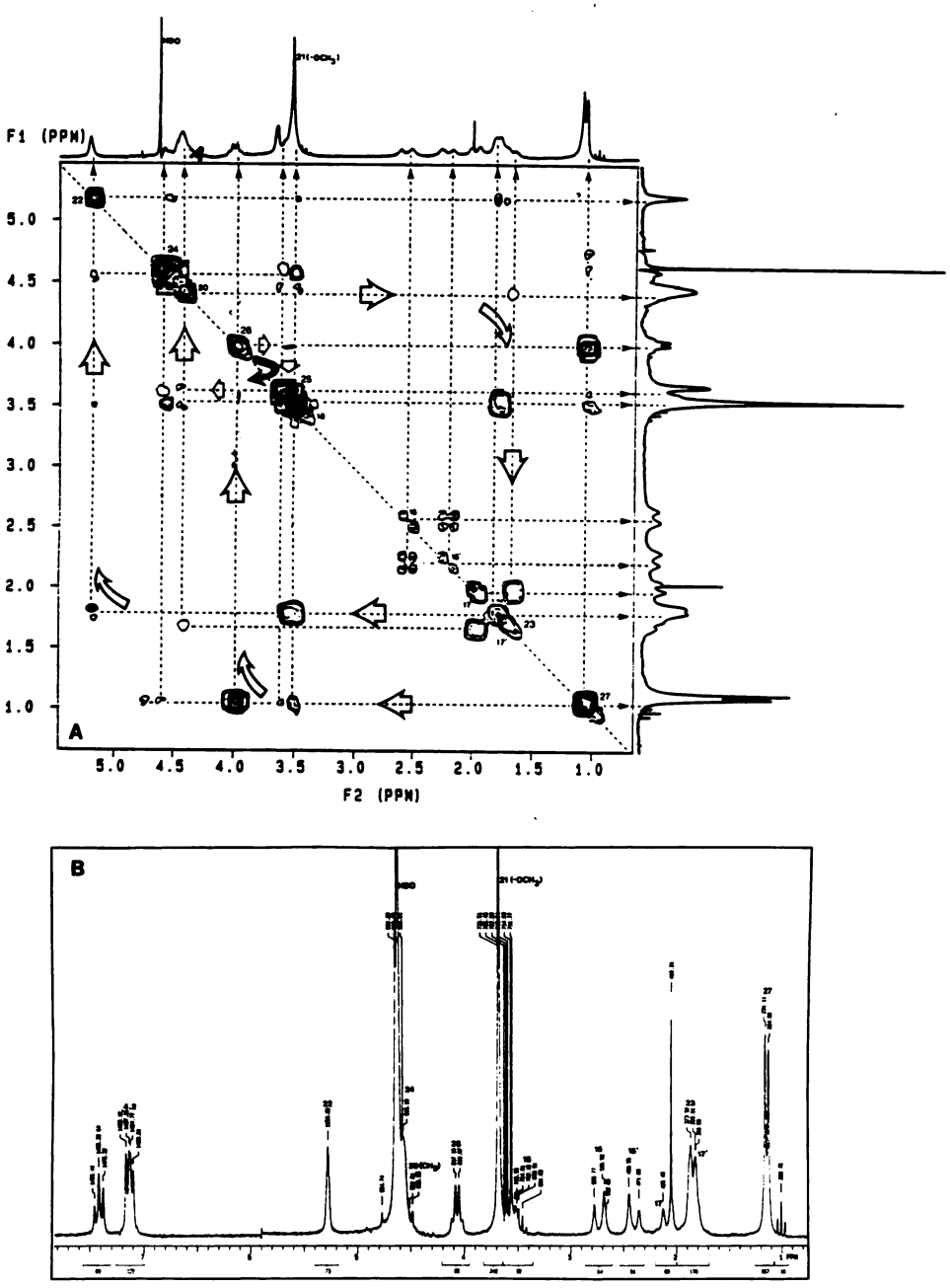

Fig. 2. ^{1}H-COSY DOX spectrum (A), showing the structural assignments referred to in the text, and the ^{1}H-FT-NMR spectrum of DOX (B), showing all structural assignments including the aromatic hydrogens.

type iron chelators, the phenolic derivatives EHPG and HBED have logK_f values of 33.9 (Fe^{3+}) and 33.6 (Ga^{3+}) and 39.7 (Fe^{3+}) and 39.6 (Ga^{3+}), respectively (25). The structurally dissimilar chelating agent used clinically for the treatment of iron overload is desferioxamine, which has logK_f values of 30.6 (Fe^{3+}) and 27.6 (Ga^{3+}) (25). Therefore, affinities for a broad range of relevant molecules are conserved. For these reasons, gallium(III) is a suitable probe for iron within the context of these experiments, where direct observation of iron(III) is not possible. A pure spectrum of gallium(III) showed no peaks and, more importantly, no interference with the magnetic field.

ADR-529 (peak assignments). Structural assignments are shown on the COSY spectrum (Fig. 3A). The assignment starts with the methyl doublet (^3J = .6.6.Hz) at 0.9 δ, correlated via a cross-peak to H_a (3.0 δ). Two cross-peaks correlate H_a to both H_b and H_c and a square connects these two nonequivalent (due to the adjacent chiral carbon) hydrogens. The H_{pip} hydrogens are all nonequivalent and give rise to a complex set of peaks around 3.4 δ. The most probable conformation (Fig. 4A), with the piperazine rings staggered, is suggested by the coupling constants (Table 1). H_a and H_b should be approximately *trans*-periplanar, with the dihedral angle near 180° and the H_a-H_b angle of approximately 60°. The Karplus relationship (22, 26) between the dihedral angle and J is consistent with the observed splittings of 4.6 Hz (~60°) and 9.5 Hz (~180°). After the proton spectra were unequivocally assigned from the COSY spectrum (Fig. 3A), these values were used to assign the carbon spectra (Fig. 3B) via the HETCOR spectrum (data not shown).

There is an obvious impurity present. The weak (10%) doublet at 1.1 δ can only be due to a methyl group adjacent to one hydrogen. Also, there are no nitrogens adjacent to the carbon to which this methyl is attached, which is the reason for the

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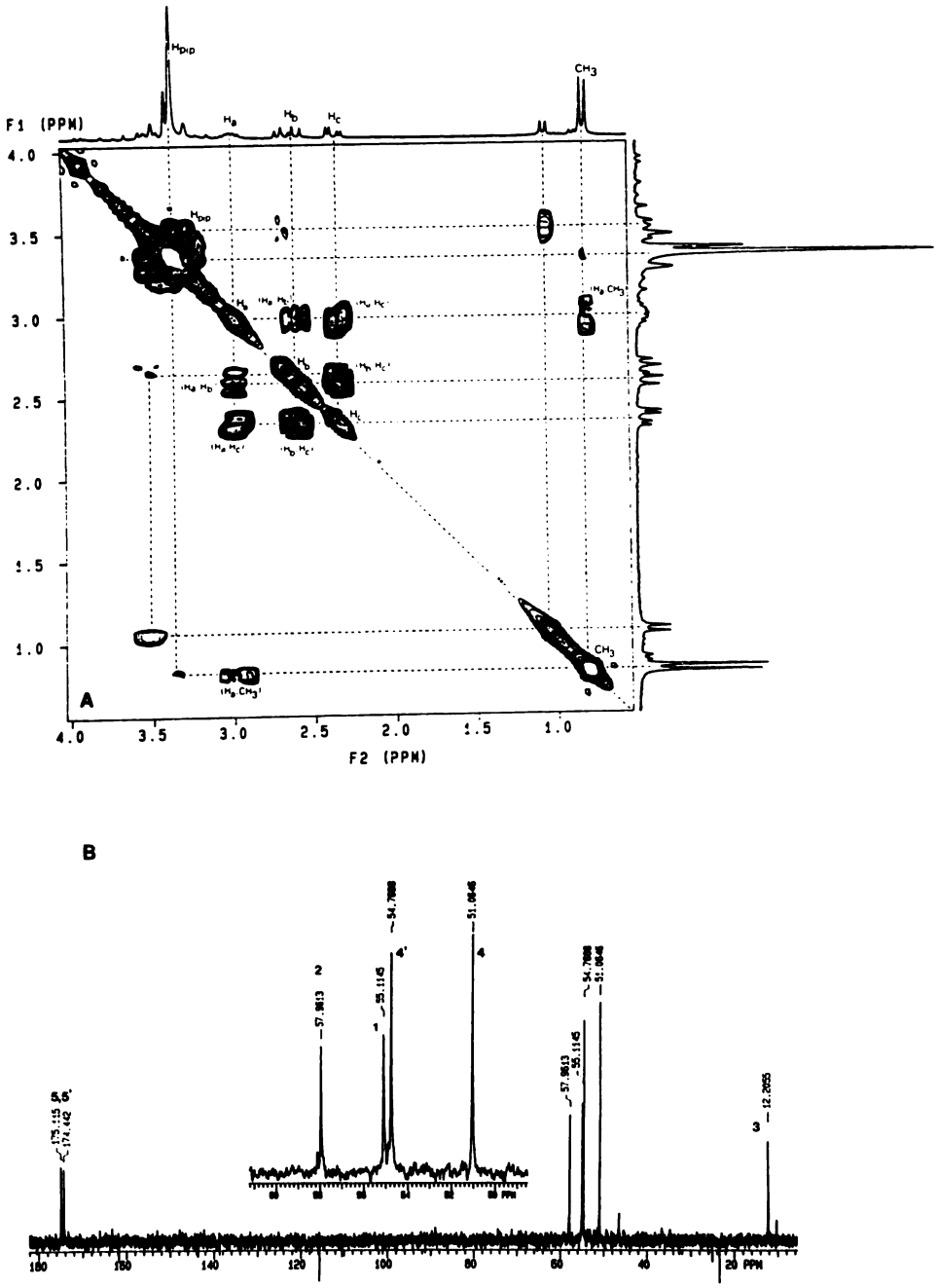

Fig. 3. ^{1}H-COSY ADR-529 spectrum (A), showing the structural assignments referred to in the text, and the ^{13}C-FT-NMR spectrum of ADR-529 (B), showing peak assignments.

downfield shift of the ADR-529 methyl peaks. This was confirmed by spectra of 1,2-propylene diol (the ADR-529 structure with hydroxyl groups replacing the diketopiperazinyl moieties) (data not shown). A strong cross-peak places this hydrogen in the complex peaks at 3.55 δ, and a second cross-peak connects back to a weak overlapped doublet at 2.7 δ. This would tend to indicate that the structure of this impurity is similar to the central hydrogens of the ADR-529 molecule and is consistent with an analog of ADR-529 differing by a hydroxyl group replacement of the diketopiperazinyl group at the carbon with the methyl group.

Gallium-ADR-529 complex. The anthracycline-iron complex has been well documented in the literature (27–34) and, therefore, data on the anthracycline-gallium complex are not presented. As previously mentioned, gallium is interchangable with iron. After addition of gallium the ADR-529 spectrum is appreciably altered (Fig. 5A). All resonances (except the impurity) are shifted to lower field, and the signals assigned to the diketopiperazinyl CH_2 groups are separated. This clearly indicates that interaction between ADR-529 and gallium has occurred, and the splittings between H_a and H_b and H_c are changed from 4.6 to 4.0 Hz and from 9.5 to 11 Hz. These values are consistent with dihedral angles of 60° and 0°, which, in addition to the field shift changes, would suggest that gallium forms a complex (Fig. 4B) locking the rings into the octahedral field of the gallium. The chemical shift changes are larger for the methyl and $H_{a,b,c}$ than for the ring hydrogens, consistent with a distance-dependant contact shift mechanism operating. These values confirm that the proposed gallium-ADR-529 complex has formed.

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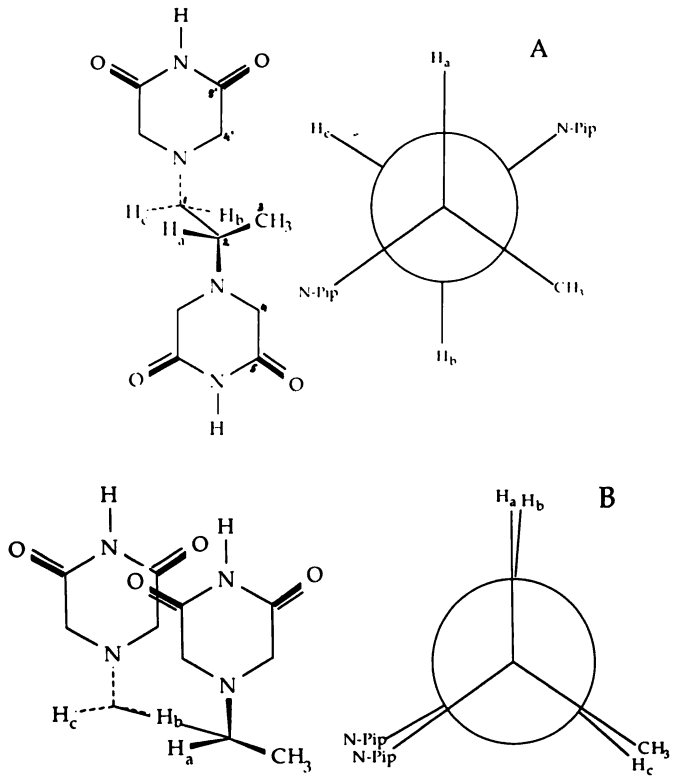

Fig. 4. Newman projection and associated structural diagrams of the proposed conformations of ADR-529 alone (A) and ADR-529 complexed with Ga^{3+} (B). These conformations have been validated by both computer modeling and these NMR experiments.

Anthracycline-Ga^{3+}-ADR-529 complex. Initial attempts to produce a complex using DOX, ADR-529, and Ga^{3+} at concentrations needed to produce clear carbon spectra with molar ratios of 2:4:5, respectively (50 mg of DOX in 0.7 ml of D_2O), produced a gel whose color was altered from either pure compound or previously attained complexes, which produced a very high level of peak broadening leading to uninterpretable results. Based on a previous theoretical paper (19), it was reasoned that EPI, by virtue of intramolecular hydrogen bonding, would have the second quinone moiety (located next to the methoxylated anthraquinone ring corner) partially blocked and, therefore, would be less prone to gel formation. This was partially true; however, the noise level was markedly raised and solubility of the formed complex was lower than anticipated.

Nevertheless, the resultant spectra (Fig. 5B) clearly show a direct interaction between the anthracycline, gallium(III), and ADR-529, which is consistent with the proposed structure. Table 1 shows that the coupling constants $J_{H_a-H_b}$ and $J_{H_a-H_c}$ are consistent with the cage-type conformation, with a slight buckling of the ethane backbone linking the two diketopiperazinyl groups. This effect is probably due to gallium having a slightly smaller ionic radius [0.62 Å and 0.64 Å for Ga^{3+} and Fe^{3+} (23), respectively], leading to the piperazinyl rings being brought together at a slightly different angle, with the keto groups closer to the anthracene substructure, thus twisting the backbone very slightly. Tables 2 and 3 show that the chemical shifts of both the anthracycline and ADR-529 alter in a manner consistent with a combined ternary complex (as described) being formed. These values clearly indicate that the proposed structure has been formed in this *in vitro* experiment. Attempts are now being made to determine whether this complex is present in the plasma of patients receiving combined chemotherapy with the two agents.

Discussion

The exact mechanism of anthracycline cytotoxicity and cardiotoxicity has not been elucidated. Data for at least four mechanisms have been published (for a recent review, see Ref. 35). Anthracyclines intercalate into nuclear DNA, leading to base pair mismatching at replication and subsequent cell death (36). Anthracyclines can also cause single- and double-strand breaks, as well as DNA-DNA and DNA-protein cross-links, through their interference with the DNA-repair enzyme topoisomerase II (37–39). In another mechanism, DOX undergoes reductive metabolic activation to various reactive intermediates, including the semiquinone free radical and the 7-quinonemethide species (40). DOX accepts a single electron from microsomal NADPH:cytochrome P-450 reductase to form the semiquinone, which rapidly autoxidizes and reduces molecular oxygen to the superoxide anion radical and, subsequently, reduced species of oxygen, including hydrogen peroxide and hydroxyl radical (41). It is now clear that iron and DOX form a complex that acts as a catalytic site for the production of several reactive species, including the hydroxyl radical and hydrogen peroxide (42). The complexed iron undergoes cyclic redox reactions alternating between Fe^{2+} and Fe^{3+}, similar to the mechanism described above and akin to the Fenton reactions of free intracellular iron. Certainly some, if not all, of these mechanisms might be important as a mechanism in cancer cell death; however, in a slowly dividing organ such as the heart, impairment of energy function appears to be the most likely cause of toxicity (43).

The observed clinical anthracycline-induced cardiomyopathy is caused by lesions on heart tissue leading to lowered pumping capacity (44). These characteristic lesions are seen on electron microscopy as predominantly those of mitochondrial destruction associated with mitochondrial membrane lysis (12). In tissue, such as heart muscle, where the antioxidant enzymes superoxide dismutase and catalase exist in low abundancies, damage due to lipid peroxidation is marked (12, 45, 46).

In a recent clinical trial, ADR-529 protected against DOX cardiotoxicity (9). The study also showed that ADR-529 does

TABLE 1
Proton coupling constants (*J*) for ADR-529 alone, with Ga^{3+}, and with Ga^{3+} and the anthracycline EPI

	ADR-529 alone		ADR-529 + Ga^{3+}		ADR-529 + Ga^{3+} + EPI	
	J	Karplus angle	*J*	Karplus angle	*J*	Karplus angle
	Hz		*Hz*		*Hz*	
H_a-H_b	9.5	180°	11.0	0°	10.9	0°
H_a-H_c	4.6	60°	4.0	60°	1.5	60°

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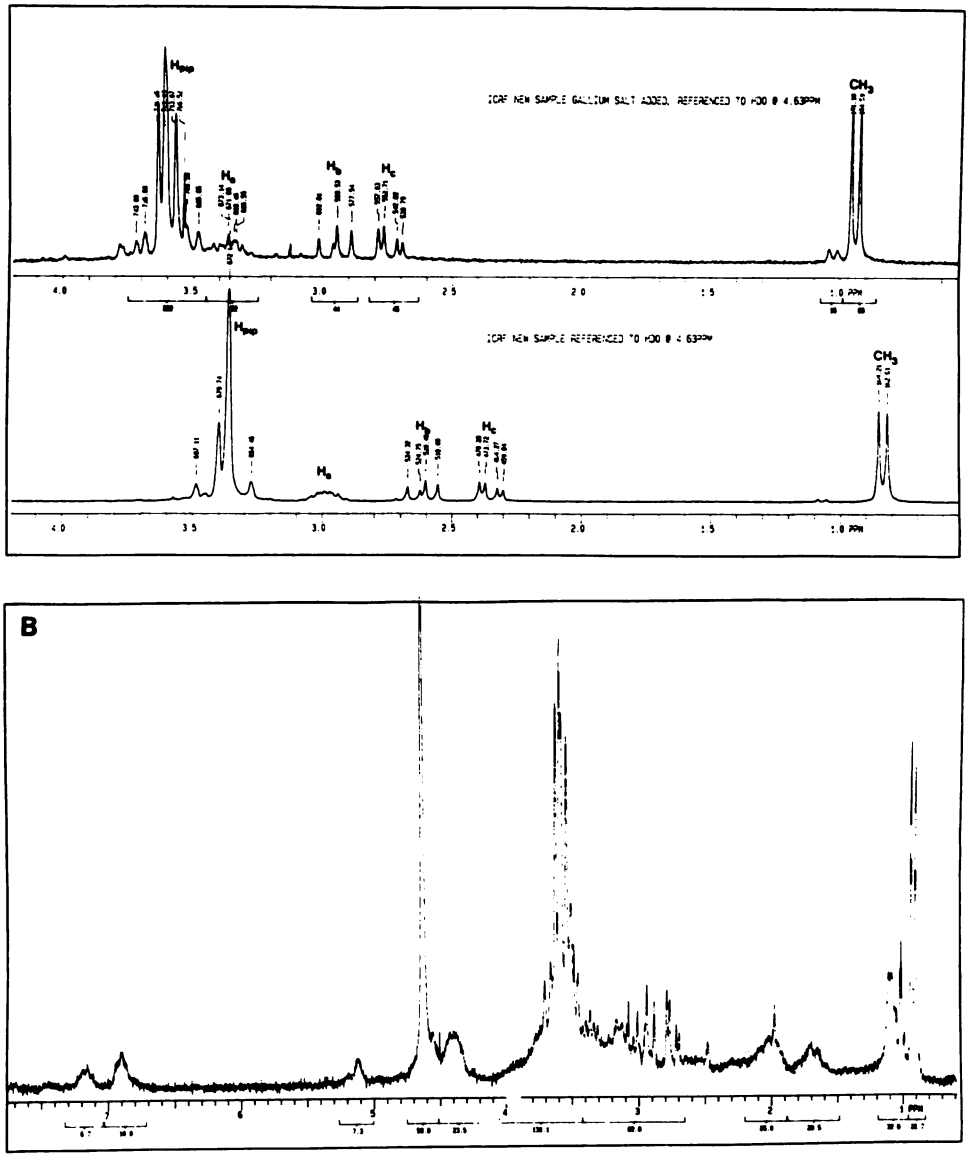

Fig. 5. Proton spectra of ADR-529 (A) with (*upper*) and without (*lower*) Ga^{3+} and the proton spectrum of the ternary ADR-529-Ga^{3+}-EPI complex (B).

TABLE 2

Proton chemical shifts for ADR-529 alone, with Ga^{3+}, and with Ga^{3+} and the anthracycline EPI

Proton label	Chemical shift[a]		
	ADR-529 alone	ADR-529 + Ga^{3+}	ADR-529 + Ga^{3+} + EPI
	δ		
H_a	2.98 (m)	3.36 (m)	3.14 (m)
H_b	2.60 (t)	2.88 (t)	2.91 (t)
H_c	2.35 (d/d)	2.74 (d/d)	2.71 (d/d)
CH_3	0.36 (d)	0.94 (d)	0.91 (d)
H_{pip}	3.36 (d)	3.59 (t)	3.36 (m)

[a] d, doublet; t, triplet; m, multiplet; d/d, split doublet, "double doublet."

not abrogate the myelosuppression and alopecia caused by DOX (9). These observations indicate that the action of the anthracycline on the heart is mediated by a mechanism different from that in rapidly dividing tissue such as bone marrow and hair follicles.

In *in vitro* studies (47), this type of membrane damage is prevented when EDTA is added to the cell medium, and thus chelation of transition metal ions might have some bearing on the mechanism. ADR-529 is a nonpolar derivative of EDTA and similarly chelates divalent and trivalent cations (27) in the cytoplasm and/or nucleoplasm.

Iron and DOX complex to form a reactive catalytic site for the production of free radical species. It has been hypothesized that ADR-529 must either chelate the iron cations, preventing the formation of the catalytic complex, or interact with the complex to reduce its ability to produce free radical species (14, 27, 45).

It has been suggested that the mechanism by which ADR-529 operates is as a free radical scavenger; however, this does not seem likely, because trials with more potent free radical scavengers showed no protection against the chronic damage against which ADR-529 protects (48–51).

Because ADR-529 complexes iron cations, some authors have suggested that the mechanism of its action is due to chelation of free iron (27). However, DOX has an extremely high affinity for iron (52–54) and, should ADR-529 deplete the localized supply of iron cations, DOX will either complex with the ADR-Fe^{3+} complex or attract and bind intracellular iron from beyond the iron-depleted zone. Even the iron-transport protein transferrin, which has a much greater affinity for iron than do any

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TABLE 3

Proton chemical shifts for DOX alone and for Ga^{3+} with the anthracycline EPI

The only relevant peak that differs in the spectra of the two stereoisomers has been labeled, to indicate that this peak differs for this reason and not because of complexation.

Proton label	Chemical shift[a]			
	Anthracycline alone		Anthracycline + Ga^{3+} + ADR-529	
	δ			
3	7.22	(t)	7.18	(d)
4	6.95	(d)	6.91	(s)
2	6.84	(d)	6.86	(s)
22	5.21	(s)	5.11	(s)
24	4.57	(s)	4.55	(s)
20	4.45	(s)	4.38	(s)
26	4.02	(d)	NP[b]	
21	3.55	(s)	3.62	(d/d)
25	3.47 (DOX)	(s)	3.57 (EPI)	(d)
18	3.44	(s)	3.45	(s)
15	2.60	(d)	NP	
15′	2.25	(d)	NP	
17	1.97	(s)	1.69	(s)
23	1.82	(d)	1.71	(s)
17′	1.71	(s)	1.63	(s)
27	1.12	(d)	1.00	(d)

[a] t, triplet; d, doublet; s, singlet; dd, split doublet, "double doublet."
[b] NP, no peak detected.

other plasma proteins, can have its Fe^{3+} extracted by DOX (55). Therefore, depletion of iron alone can not adequately explain the observed cardioprotection, especially in view of the ample supply of available iron in humans and clinical studies (20, 21) that have shown that the anthracycline has the lowest concentration and is, therefore, the limiting reactant in each case.

One possible explanation is that ADR-529 has an effect on the Fe^{3+}-DOX complex, which reduces its ability to produce free radicals. For example, ADR-529 can be hydrolyzed to form the compound ICRF-198 [(±)-*N,N′*-dicarboxamidomethyl-*N,N′*-dicarboxymethyl-1,2-diaminopropane] (27–29). Another investigator has suggested that ICRF-198 abstracts the iron from the complex (29). In these isolated *in vitro* studies (27–29), both ADR-529 and its hydrolyzed equivalent ICRF-198 proved effective in protecting against Fe^{3+}-Adriamycin-induced inactivation of cytochrome *c* oxidase activity of submitochondrial particles. The studies demonstrated that ICRF-198 completely removed Fe^{3+} from the complex, whereas ADR-529 did not seem to extract the complexed iron. Under these conditions, it would seem that the complex was not reformed once dissociated; however, in an *in vivo* environment where iron supply is plentiful, this is unlikely to be the mechanism of cardioprotection because, if ICRF-198 were to remove the complexed iron, then DOX would probably replace it with ease from intracellular free iron or from iron-proteins, due to the very high complex formation constants (56, 57). In contrast, we have proposed that ADR-529, DOX, and iron cations form a ternary complex (as in Fig. 1B) that leads to steric hinderance around the active catalytic site and halts the production of free radicals at that site, thereby reducing the cardiomyopathy associated with DOX administration by this means. Our data are consistent with the formation of such a ternary complex.

ADR-529 in aqueous solution can exist in a number of conformations. From modeling studies, the lowest energy state is with the two diketopiperazinyl groups at maximum separation. The NMR data confirmed this prediction of the preferred solution conformation for pure ADR-529. The nitrogens in the diketopiperazinyl groups exist in a planar sp_2 hybrid form, with the lone pair being evenly distributed along the plane. As ADR-529 approaches the octahedrally complexed iron cation, its conformation could alter, such that it would form a cage-like structure. The lone pair on each of the nitrogens would draw itself together, changing the sp_2 trigonal planar nitrogen to an sp_3 tetrahedral one with the lone pairs pointing into the cage, allowing the nitrogens to displace the monodentate water ligands to form a tetradentate square-planar complex with waters occupying the last two positions on the octahedral iron cation (Fig. 1B). Again, the NMR data confirmed this prediction.

Iron cations have been shown to form a complex with DOX (58), as in Fig. 1C. In this complex, two positions of the octahedral complex would be associated with DOX binding and the other four would form a square planar complex, with water acting as monodentate ligands. Thus, a σ bond is established between the hydroxyl oxygen and the Fe^{3+}, along the hybrid d_2sp_3 orbital primarily associated with the prehybridized d_{z^2} orbital, and a dative bond is formed, with the lone pair of the carbonyl being donated into the orbital primarily in the prehybrid $d_{x^2-y^2}$ lobe. At this stage, water molecules occupy the other four positions, with the lone pairs of each water oxygen being directed into the empty hybrid orbitals of the Fe^{3+}.

Our proposal is that ADR-529 displaces the water ligands and becomes a tetradentate ligand completing an octahedral complex centered on the iron cation, in the same manner as already described for ADR-529-Fe^{3+} complexes. This complex would be expected to be stable and to sterically prevent the formation of radical species from the Fe^{3+}-DOX site. The NMR spectra are consistent with this proposal, with respect to the formation of this complex. *In vivo*, anthracycline-complexed iron undergoes continuous redox cycling with changes of ionic radius of 0.64 Å to 0.74 Å from Fe^{3+} to Fe^{2+}, respectively. Modeling shows that the extra radial length of Fe^{2+} can fit within the cleft produced in the ternary complex; however, it is plausible that the ternary complex is more likely to form during the Fe^{3+} part of the cycle.

The value of this knowledge is that, apart from our increased knowledge of *in vitro* complex formation, we are now aware of a possible *in vivo* mechanism of drug interaction that warrants further study.

These data support the notion (14) that single agents could be rationally designed with improved toxicological profiles, based on greater knowledge of therapeutic drug interactions from *in vitro* studies. To this end, this study has demonstrated the feasibility of readily forming such a complex under conditions compatible with the physiological environment of the *in vivo* target organ (i.e., myocardial tissue). Direct testing of this hypothesis in an *in vivo* model is presently beyond the technical capacities of existing methods. This is due to the signals from such a complex being too low to detect by present magnetic resonance imaging technology, and even NMR studies on heart biopsies (whether human or mice) are subject to substantial masking by co-resident materials, thus limiting studies to *in vitro* models and computer simulations that mimic some biological parameters.

Acknowledgments

M.M.S. thanks Drs. Ted Lloyd, Jeff Dyson, Tracy Nero, Mark Searle, and Mark Smythe, School of Pharmaceutical Chemistry, Victorian College of Pharmacy and Peter MacCallum Cancer Institue, for their assistance, the members of the Molecular Design Society (Australia) for their interest, and especially Dr.

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Colin Rix, Department of Applied Chemistry, RMIT, for his assistance and suggestion to use the gallium probe to overcome the problems associated with NMR of iron(III) salts.

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