Citation for published version:
Young, B 2015, 'Synthesis and Biological Studies on 'Smart' Iron Chelator Molecules', Ph.D., University of Bath.

Publication date:
2015

Document Version
Publisher's PDF, also known as Version of record

Link to publication

University of Bath

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CHAPTER 5: RESULTS AND DISCUSSION (PART IV)

S-caged thiosemicarbazone iron chelators

5.1. Introduction

Since the discovery of 3-AP (13, Figure 5.1) and the link between its iron-chelating and potent anti-proliferative activities, a number of novel, lipophilic ICs have been synthesised based on the thiosemicarbazone backbone. An example is the di-2-pyridyl ketone thiosemicarbazone (DpT) series of ICs (see Figure 1.14,) which were developed by Richardson and colleagues who subsequently reported their potent cytotoxic activity.\cite{86}. Compound Dp44mT (15a, Figure 5.1) was found to possess the most potent anti-proliferative activity across a range of tumour cell lines, with an IC\textsubscript{50} of 0.03 \(\mu\)M in SK-N-MC neuroepithelioma cells \cite{179} compared to 0.3 \(\mu\)M for NIH and 0.26 \(\mu\)M for 3-AP.

![Figure 5.1. 3-AP (Triapine\textsuperscript{\textregistered}, 13) and Dp44mT (15a): two examples of thiosemicarbazone ICs.](image)

Significantly, Dp44mT has been found to possess a degree of selective antitumour activity, as its antiproliferative effects are considerably lower in certain non-tumourigenic or 'normal' cell lines, such as MRC-5 fibroblasts. It was also reported that 15a was active against etoposide-resistant cell lines, and was in fact more active in vinblastine-resistant tumours compared to vinblastine-sensitive cells, thus demonstrating the potential that IC-based chemotherapy could have in overcoming resistance to conventional cytotoxics.\cite{180} Studies on the \textit{in vivo} administration of Dp44mT to mice have shown marked antitumour efficacy, and although generally well-tolerated, cardiac fibrosis was observed with higher doses.

Following on from the DpT series of compounds, the BpT series of ICs were developed where the non-coordinating pyridyl moiety on the molecule is replaced with a phenyl ring, which would further increase the lipophilicity of the molecule. It was also postulated that by replacing the electron-withdrawing pyridyl group with a phenyl group,
the redox-potential of the Fe-complex may be reduced, which would thus enhance the extent of redox-cycling and increase the cytotoxicity of the compound.\textsuperscript{[87]} It was found that these compounds, known as the 2-benzoylpyridine thiosemicarbazones (BpT) possessed higher cytotoxicity than their DpT counterparts, with Bp4eT, 16d eliciting the most potent effect with an IC\textsubscript{50} of 2 nM. As with the DpT series, the antiproliferative effects of the BpT series were found to be selective for cancer cells over ‘normal’ cell lines, and a recent in vivo study with Bp44mT has shown potent anti-tumour efficacy when administered orally to mice.\textsuperscript{[181]} Significantly, the cardiac toxicity associated with Dp44mT was not observed with Bp4eT, although mild hepatotoxicity was observed.

The significant toxicity observed with 3-AP (Triapine\textsuperscript{®}) during clinical trials as an anti-tumour agent was a major contributing factor for its perceived clinical failure, and despite the promising in vivo results observed with 15a and 16d, adverse effects resulting from iron depletion in healthy tissue remains a potential stumbling block in the development of these potent iron chelators.

The development of caged thiosemicarbazones represents a novel strategy to attenuate the potent cytotoxicity of compounds such as Dp44mT, so that the desired biological effects are harnessed to achieve anti-proliferative activity in more highly targeted fashion. This concept bears similarities to PDT, where selective exposure of diseased tissue to a potent photosensitiser can easily be achieved by an external light source. CICs offer a similar therapeutic strategy, which has potential applications in the treatment of skin cancers and other dermatological disease states such as psoriasis.
5.2. Synthesis of S-caged DpT iron chelators

As the DpT series of ICs do not possess an oxygen donor atom, attachment of a PRPG at either nitrogen or sulfur is necessary to furnish a thiosemicarbazone-CIC that is temporarily blocked from binding to iron. It has already been shown that sulfur-containing compounds which possess a thione group can undergo S-alkylation with soft electrophiles as illustrated by the S-methylation of 167 to give 168 (Scheme 5.1). Attachment of the NPE caging group to the thioamide sulfur was therefore considered to be a feasible synthetic strategy.

\[ \text{Scheme 5.1. S-alkylation of thiosemicarbazide derivative 167 as described by Ouyang et al.}^{[182]} \]

Reagents and conditions: Mel, acetone, reflux, 4 h, 95%.

Synthesis of the DpT compounds 12a-c was achieved by condensation of di-2-pyridyl ketone (169) with the corresponding N₄-substituted thiosemicarbazide, or in the case of 15c, thiobenzhydrazide 59 as shown in Scheme 5.2. Isolation of the pure compounds was accomplished by recrystallisation from EtOH or aqueous EtOH to give 15a-c in 34-63% yield.

\[ \text{Scheme 5.2. Preparation of DpT iron chelators from the corresponding thiosemicarbazides. Reagents and conditions: a. 4,4-dimethyl-3-thiosemicarbazide, EtOH, reflux, overnight; b. 4-phenyl-3-thiosemicarbazide, EtOH, reflux, 7 h; c. 59, EtOH, reflux, overnight; d. 4-ethyl-3-thiosemicarbazide, EtOH, overnight.} \]

When these conditions were employed for the 4-ethyl-3-thiosemicarbazide however, no reaction was observed to take place and compound 15d was not observed by TLC. It
should be noted that Richardson et al. have reported the successful preparation of 15d and other simple N-alkyl derivatives using this methodology.[86]

With three DpT iron chelators now in hand, alkylation at sulfur was attempted using the conditions described by Ouyang et al.[182] This involved simply heating the complete DpT compound 15a or 15b with NPE-bromide 49 in acetone to give the corresponding S-caged DpT compounds 170a and 170b in modest yields of 29% or 28% respectively (Scheme 5.3). TLC analysis following overnight reaction showed a clean conversion to alkylated products, and the presence of unreacted starting materials, which accounts for the relatively low yields.

![Scheme 5.3](image)

**Scheme 5.3.** S-alkylation of DpT compounds Dp44mT 15a and Dp4pT 15b with the NPE group to give 170a-b. *Reagents and conditions:* 49, acetone, reflux, overnight. See table 5.2 for yields.

This synthetic route was also used to prepare DEACM-caged DpT derivative, 171 (Scheme 5.4); however this reaction proceeded much more efficiently compared to alkylation with NPE, with complete consumption of starting material by TLC analysis and isolation of the expected product in 90% yield. DEACM-bromide 73 being a primary halide, would indeed be anticipated to be much more reactive towards S_N2 nucleophilic attack by the thioamide sulfur than NPE-bromide, a secondary halide.

![Scheme 5.4](image)

**Scheme 5.4.** S-alkylation of Dp4pT with the DEACM caging group to give 171

*Reagents and conditions:* 73, acetone, reflux, 8.5 h, 90%.
5.3. Attempted synthesis of Bp4eT

As discussed above, efforts to prepare the 4-ethyl DpT derivative (13d) as shown in Scheme 5.2 were unsuccessful, and when 4-ethylthiosemicarbazide was instead heated with 2-benzoylpyridine 172 in an attempt to give Bp4eT (16d, Scheme 5.5), the same result was observed and no reaction was found to occur by TLC.

![Scheme 5.5. Attempted synthesis of Bp4eT 16d. Reagents and conditions: 4-ethyl-3-thiosemicarbazide, EtOH, reflux, 18 h.](image)

It was later discovered that Richardson and colleagues describe a separate set of synthetic conditions to prepare the BpT series of compounds, which differs slightly from the method used to prepare the DpT iron chelators. These included the use of a catalytic amount of acetic acid, and addition of the thiosemicarbazide as an aqueous solution instead of as a dry powder. When these conditions were employed, TLC analysis showed a small degree of reaction progression; however the expected product was only isolated in 5% yield (Scheme 5.6).

![Scheme 5.6. Preparation of Bp4eT 16d using revised conditions. Reagents and conditions: 4-ethyl-3-thiosemicarbazide, AcOH, EtOH/H2O (1:1), reflux, overnight, 5%.](image)

Although it is evident that the addition of AcOH as a catalyst is helpful, its specific role in this reaction is not currently completely understood. It is probable that it acts to protonate the hydroxyl anion on the tetrahedral intermediate which is formed following
nucleophilic attack by the hydrazide nitrogen, thus accelerating the elimination of water to give the Schiff base (Scheme 5.7 shows an example). It is unclear however why an acid catalyst is required for this reaction, whereas condensation with the other thiosemicarbazides occurs without the need of an acid catalyst.

Scheme 5.7. Mechanism for the condensation of a thiosemicarbazide with diarylketone 172, and its facilitation by an acid catalyst (H⁺)

Doubling the amount of acetic acid or extending the reaction time made no difference to the reaction outcome according to TLC analysis. It was thought that removing water from the reaction medium might help to drive the reaction forward based on the principle that water is actually generated by condensation of the thiosemicarbazide and diarylketone. However, when water was omitted from the reaction above, and molecular sieves added to remove water generated from the reaction, there was again no improvement seen by TLC analysis.

The fact that condensation of an arylketone with other substituted thiosemicarbazides had been found to proceed without any significant problems suggested that the 4-ethyl thiosemicarbazide derivative 173 or other simple mono-N-alkyl derivatives, might be peculiarly unreactive to simple condensation. An alternative strategy therefore was considered, whereby the thiosemicarbazide 173 was ‘caged’ with the NPE group prior to condensation with arylketone 172, to give the final desired Schiff base. S-alkylation of thiosemicarbazide 173 was therefore attempted using the conditions described
previously; however, even though the expected product 174 could be identified by NMR analysis, a mixture of compounds was obtained, including a considerable amount of unchanged starting material, which could not be separated by column chromatography.

When the reaction was repeated using another higher boiling polar solvent, acetonitrile, no improvement in conversion was apparent by TLC analysis.

5.4. Fe-complex experiments

With the S-caged DpT compounds 170a-b in hand, it was hoped that these compounds would not form complexes when mixed with an iron salt. To demonstrate that iron chelating activity is abolished in the S-caged compounds, an iron complex with the parental chelator Dp44mT (15a) was therefore first generated as a control according to the method reported by Richardson (Scheme 5.9).[86]
The UV absorbance spectra of the isolated complex 175, and Dp44mT itself (15a) was measured at a concentration of 20 μM in EtOH (Figure 5.3, A) The S-caged Dp44mT compound 170a, was subjected to the same conditions as shown in Scheme 5.9, and the UV spectra of the reaction mixture was also measured (Figure 5.3, B).

![Graph A](image1.png) ![Graph B](image2.png)

**Figure 5.3.** UV absorption spectra of ‘naked’ Dp44mT and its iron complex (A) and the corresponding NPE-caged compound 170a (B).

It can be seen that the uncaged IC 15a undergoes a considerable bathochromic shift upon complexation with iron, with the observed \( \lambda_{\text{max}} \) value for the Dp44mT-Fe complex being consistent with that stated in the literature.\[^{[86]}\] The S-caged compound appears to have the same absorption profile at wavelengths above 320 nm both in the absence and presence of iron, however it was surprising to observe a dramatic increase in absorption around 270 nm when the caged compound was mixed with iron. Based on these results alone it is difficult to account for this observation, although weak complexation of iron in a bidentate fashion through the pyridyl and imine nitrogen donor atoms might be responsible. However complexation with iron for Dp44mT appears to red-shift the absorption maxima (as seen in A), and this is not observed in figure B for the caged compound, rather the intensity of absorption is simply increased. Further work to elucidate this phenomenon is therefore required.
5.5. Decaging experiments

5.1. UV absorption spectra

The UV absorbance profile for the NPE and DEACM-caged derivatives of Dp4pT (\textbf{170b} and \textbf{171} respectively) is shown in Figure 5.4. As one would expect, consistent with other DEACM-caged compounds the CIC \textbf{171} displays a high level of absorption in the near-UV region compared to the NPE analogue; however both compounds appear to absorb comfortably within the UVA region, as does the NPE-S-caged Dp44mT derivative \textbf{170a} (not shown).

![Figure 5.4. UV absorbance spectra of the NPE and DEACM-caged derivatives of Dp4pT in EtOH.](image)
5.2. Single dose UVA decaging experiments

The uncaging profile of NPE-caged Dp4pT 170b is shown below in Figure 5.5. It is apparent that at a dose of 250 kJ/m², the compound only undergoes partial photolysis (B); however the expected iron chelator (C) and NPK photofragment (D) appear to have been released as anticipated. This is confirmed by co-injection of the irradiated sample with Dp4pT and NPK (E and F respectively). Although this compound has an absorption maxima within the UVA range at 345 nm, the level of absorption at this wavelength is relatively modest ($\varepsilon = 11086$ L mol⁻¹ cm⁻¹ at 345 nm).

![HPLC chromatograms](image)

**Figure 5.5.** HPLC chromatograms of NPE-Dp4pT 170b and its related photoproducts, depicting the caged compound (A) and the UVA irradiated (250 kJ/m²) compound, magnified for clarity (B). Also shown for reference is the ‘naked’ Dp4pT molecule (C) and NPK (D) as well as co-injection of the irradiated sample with either Dp4pT (E) or NPK (F).
The decaging profile of NPE-Dp44mT \((170a)\) is shown in Figure 5.6, where it can be seen that, in contrast to NPE-Dp4pT, photolysis appears to occur to a much greater extent, although a small degree of intact CIC appears to remain following irradiation \((B)\). This observation may be reflective of this compound absorbing more strongly within the UVA range compared its Dp4pT derivative \((\lambda_{\text{max}} = 337.3 \text{ nm}, \varepsilon = 20029 \text{ L mol}^{-1} \text{ cm}^{-1})\).

The released photoproduct signals are consistent with those of the expected compounds, namely Dp44mT \((C)\) and NPK \((D)\), which was further confirmed by co-injection of the irradiated sample and NPK \((E)\). Despite this, the generation of additional, unexpected photoproducts appears to be evident \((B)\), for example the signal with an \(R_t\) value of 5.02 min which has not been identified.

**Figure 5.6.** HPLC chromatograms of NPE-Dp44mT \(170a\) and its related photoproducts, depicting the caged compound \((A)\) and the UVA irradiated \((250 \text{ kJ/m}^2)\) compound \((B)\). Also shown for reference is the ‘naked’ Dp44mT molecule \((C)\) and NPK \((D)\) as well as co-injection of the irradiated sample with NPK \((E)\).
Irradiation of the DEACM-caged Dp4pT compound (171) however appears to generate an array of photoproducts which cannot be identified (Figure 5.7, B), as none of the signals observed correlate with the 'naked' Dp4pT IC 15b, suggesting it is not photoreleased as anticipated. It is interesting that this is also observed with the corresponding O-caged DEACM compounds 75 and 78 (see chapter 2) which have similar HPLC profiles following UVA irradiation.

Figure 5.7. HPLC chromatograms of DEACM-caged Dp44mT 171 and its related photoproducts, depicting the caged compound (A) and the UVA irradiated (250 kJ/m²) compound (B). Also shown for reference is the 'naked' Dp4pT molecule (C).
5.5. Biological results

Figure 5.8 shows the growth-inhibitory effect of Dp44mT 15a on HaCaT cells when incubated for 24, 48 or 72 hours at a concentration of 0.1, 0.2 or 0.4 µM. Surprisingly, the toxicity of this compound does not appear to be dose-dependent, and around 50% of inhibition is achieved with all three concentrations at 72 h. There is however a clear association between incubation time and antiproliferative effect. These results do nevertheless demonstrate the potent cytotoxicity of this compound in the HaCaT cell line, as it is the only compound tested in this study where >50% cellular enzymatic activity is inhibited at concentrations as low as 0.1 µM.

![Bar chart showing enzymatic activity (%)](Image)

**Figure 5.8.** MTT assay: toxicity of Dp44mT 15a on HaCaT cells after incubation for 24, 48 or 72 h (n = 3-8)

* * * p < 0.05, significant difference between value and corresponding untreated control.
Δ Δ Δ p < 0.05, significant difference between value and corresponding treatment at 48 h
§ § § p < 0.05 significant difference between value and corresponding treatment at 24 h.

The related compound Dp4pT 15b (Figure 5.9) is also seen to elicit potent growth inhibition, with >50% cellular enzyme activity abolished at concentrations of 1 µM or below following a 72 h incubation time. This makes it less toxic than the Dp44mT derivative in the HaCaT cell line, which is consistent with the results reported by Richardson [86] who describe the Dp44mT derivative being the most potent analogue of this series in other tumourigenic cells. Unlike Dp44mT however, there is a clear dose-dependent toxicity profile observed with this compound.
Given the poor decaging profile seen with NPE-Dp44mT, this compound was not tested in this cell line; however the toxicity of NPE-Dp4pT (170b) at various concentrations in the absence and presence of UVA radiation was evaluated (Figure 5.10).

![Graph of Enzymatic activity (%)](image)

**Figure 5.9.** MTT assay: toxicity of Dp4pT 15b on HaCaT cells after incubation for 24, 48 or 72 h (n = 3-8)

- *p < 0.05, significant difference between value and corresponding untreated control.
- Δ p < 0.05, significant difference between value and corresponding treatment at 48 h
- § p < 0.05 significant difference between value and corresponding treatment at 24 h.

**Figure 5.10.** MTT assay: toxicity of Dp4pT and its NPE-caged analogue 170b (+/- UVA, 250 kJ/m²) on HaCaT cells 72 h post-treatment (n = 3).

- * p < 0.05, significant difference between value and corresponding untreated control.
- # p < 0.05, significant difference between value and corresponding treatment with Dp4pT.
It is evident from these data that at concentrations above 0.2 μM, the caged compound NPE-Dp4pT 170b displays significantly higher cytotoxicity than the ‘naked’ IC molecule in a highly dose-dependent fashion, and that upon irradiation with a UVA dose of 250 kJ/m², this toxicity is attenuated and is comparable to that seen with ‘naked’ Dp4pT. The decaging profile for this compound (Figure 5.5, section 5.4) has shown that this compound undergoes only partial photolysis at this dose of UVA however, and so the biological effects seen following irradiation cannot be attributed to that of the Dp4pT molecule alone.

A very similar biological profile is seen with the ‘naked’ and DEACM-caged Dp4pT compound 171 (Figure 5.11), where once again the caged compound appears to exert a higher level of antiproliferative activity than the parent chelator molecule, and toxicity is diminished upon irradiation with UVA. Given the rather ‘chaotic’ decaging profile of this compound however (Figure 5.7) it is unclear what photoproducts are released and thus what may be responsible for this observation.

![Enzymatic activity (%)](image)

**Figure 5.11.** MTT assay: toxicity of Dp4pT, and its DEACM-caged analogue 171 (+/− UVA) on HaCaT cells at 1.0 μM (n = 3).

* p < 0.05, significant difference between value and corresponding untreated control.
Δ p < 0.05, significant difference between value and corresponding treatment at 48 h
§ p < 0.05 significant difference between value and corresponding treatment at 24 h
The BrdU assay was performed on the parent iron chelators Dp44mT and Dp4pT (15a and 15b respectively) with the results shown in Figure 5.12, which shows that a lower proportion of cells treated with Dp44mT incorporate BrdU compared to Dp4pT. This suggests that Dp44mT exerts a more potent growth inhibitory effect than Dp4pT, as the extent of DNA synthesis in Dp44mT-treated cells is almost half that seen for Dp4pT treated cells. This correlates well with the results seen in the MTT assay, where Dp44mT appeared to possess a more potent cytotoxic effect in the HaCaT cell line.

**Figure 5.12.** BrdU assay: toxicity of 15a-b on HaCaT cells 72 h post treatment (n = 1).