Discovery of selective, antimetastatic and anti-cancer stem cell metallohelices via post-assembly modification†

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Helicates and related metallofoldamers, synthesised by dynamic self-assembly, represent an area of chemical space inaccessible by traditional organic synthesis, and yet with potential for discovery of new classes of drug. Here we report that water-soluble, optically pure Fe(II)- and even Zn(II)-based triplex metallohelices are an excellent platform for post-assembly click reactions. By these means, the in vitro anticancer activity and most importantly the selectivity of a triplex metallohelix Fe(II) system are dramatically improved. For one compound, a remarkable array of mechanistic and pharmacological behaviours is discovered: inhibition of Na+/K+ ATPase with potency comparable to the drug ouabain, antimetastatic properties (including inhibition of cell migration, re-adhesion and invasion), cancer stem cell targeting, and finally colonosphere inhibition competitive with the drug salinomycin.

The addition of new covalent bonds to supramolecular assemblies by post-assembly modification (PAM) may be used for various purposes including locking-down dynamic structures, triggering structural transformations, or simply late-stage derivatisation to introduce new functional groups. The latter is attractive to us since it may facilitate phenotypic discovery, property optimisation or the elucidation of structure/activity relationships, all without the need for extensive pre-assembly ligand synthesis. Further, we may include functional groups that are incompatible with the self-assembly. However, such reactions must be clean and efficient under mild conditions, and we note that the lability or reactivity of many metallosupramolecular structures means that application of the otherwise extremely versatile copper-catalysed azide/alkyne cycloaddition (CuAAC) may be hampered by deleterious reactions such as displacement of the original metal by copper from the catalyst. In addition the new triazole units formed may bind to metals.

We report here that our self-assembled optically pure, water-soluble triplex metallohelices provide excellent molecular frameworks on which to perform such late-stage modification. Through this chemistry we have discovered compounds with excellent potency against a panel of cancer cell lines, with enantioselectivity reflected in cell cycle studies, plus enhanced selectivity with respect to a panel of non-cancer cell lines in vitro. One selected compound displays a remarkable array of properties: antimetastatic (inhibition of cell migration, re-adhesion and invasion), cancer stem cell targeting, and colonosphere inhibition competitive with the drug salinomycin.

Introduction

Lehn envisaged in the original report that helicates – self-assembling multitemetallic coordination compounds – may find uses in biochemistry. Indeed, while their underlying chemistry is very different to that of the small cationic α-helical peptide units that are deployed in nature for e.g. signalling, structural and host-defence roles, some such metallofoldamers have similar dimensions and charge. With this in mind we have developed several classes of water-compatible, optically pure metallohelix compounds, each of which has unique properties, including a growing list of peptide-like behaviours: binding of DNA motifs, anticancer activity, and the inhibition of e.g. amyloid-β aggregation, enzyme activity and ice recrystallization. Thus, while we cannot expressly mimic the exquisite architectures of natural peptides, we are motivated to seek methods by which diverse metallohelices might be rapidly accessed and new biological properties discovered and optimised.
Mechanistically, the compound does not induce apoptosis but appears to inhibit Na+/K+ ATPase activity with potency comparable to the drug ouabain.

Results

Click synthesis of new metallohelices

We recently synthesised ranges of optically pure water-soluble metallohelices in which the ligand strands run in opposing directions (the "head-to-head-to-tail" or HHT isomers). Of these we selected the system based on \([\text{M}_2\text{L}_3]^{+}\) (Fig. 1, R = H) since it possesses an appealing facially amphipathic architecture. The new metallohelix enantiomers \([\text{M}_2\text{L}_3]^{+}\) (M = Zn, Fe) with perchlorate and chloride counter-ions respectively, synthesised with sub-components 1 and 5-(propargyloxy)picolinaldehyde (2), are decorated with three chemically inequivalent alkyne substituents on one face of the structure.

The \(^1\)H NMR spectra have several unusual features that confirm the topologically asymmetric structure [Fig. 1a]. In \([\text{Zn}_2\text{L}_3]^{[4]}\text{ClO}_4\) at 293 K, three spectroscopically unique ligand environments give rise to imine singlets \(H^4\) at 9.26, 9.17 and 8.80 ppm. Two of the bpy protons \(H^3\) appear in the same region (9.22 and 9.17 ppm) as a result of intramolecular hydrogen bonds – see dotted lines in structures of Fig. 1. The third bpy proton \(H^5\) with no such interaction was found at 8.39 ppm. Similarly, the two sets of ring protons \(H^4\) and \(H^5\) arising from pendant phenyls taking part in bifurcated \(\pi\)-stacks with coordinated bpy units, appear at 6.80 and 5.90 ppm. The third phenyl group that is instead \(\pi\)-stacked to a coordinated pyridine has \(H^6\) and \(H^7\) resonances at more conventional chemical shifts of 7.11 and 6.96 ppm. At lower temperatures these signals begin to broaden, consistent with slowing of phenyl group de-coordination/rotation (ESI Fig. S3†). The rather rigid arrangement of the ligand strands leads to six distinct resonances for \(H^3\), clustered at 4.42–4.10 ppm (apparent triplets) and 3.63–3.47 ppm (approximately doublets of doublets). Three alkyne proton singlets \(H^8\) appear at 3.0–2.8 ppm.

In the same \(^1\)H NMR spectrum of Fig. 1 the small singlet at 8.7 ppm is assigned to the HHH isomer of this compound – the three-fold symmetric compound where all three strands run in the same direction – and on this assumption we estimate the selectivity HHT:HHH to be ca. 99%. Other small peaks consistent with the presence of this minor isomer can be seen in the baseline. At 6.8 ppm a doublet is tentatively assigned to protons of type e in the HHH isomer. It is interesting to note the absence of a triplet for type d protons in the region 6.4–6.8 ppm in this minor component; no such signal is expected since there is no phenyl-bpy \(\pi\)-stack in the HHH isomer. Similarly, no minor doublets for type e protons are expected around 6 ppm. As such, the appearance of these minor isomer peaks corroborates our assignments for the major isomer.

Reactions employing a range of CuAAC conditions were explored and it was found that heating \([\text{M}_2\text{L}_3]^{+}\) (M = Zn, Fe) with benzyl azide in the presence of catalytic copper(i) iodide for 18 h cleanly gave \([\text{M}_2\text{L}_3\text{Bz}]^{+}\). The disappearance of the alkyne resonances in the \(^1\)H NMR spectra of \([\text{Zn}_2\text{L}_3]^{+}\) at ca. 3 ppm (H\(^\beta\)) [Fig. 1b] and 78/77 ppm (C\(^2\)/C\(^6\)) (ESI Fig. S4†) demonstrates that the reaction progresses to completion. Upon formation of the triazole moiety, three new proton singlets (H\(^\gamma\)) are evident at ca. 8 ppm and new quaternary carbon resonances (C\(^6\)) at ca. 142 ppm are observed. In addition, three new singlets at ca. 5.6 ppm are observed in the \(^1\)H NMR spectra of both \([\text{Zn}_2\text{L}_3]^{+}\) and \([\text{Fe}_2\text{L}_3]^{+}\) and \([\text{Fe}_2\text{L}_3\text{Bz}]^{+}\) (and \(^1\)C at ca. 55 ppm), due to the addition of the benzyl methylene group (H\(^\delta\)), as well as new resonances due to the benzyl ring in the aromatic region. The NMR signals corresponding to the imine, bipyridyl and phenyl units remain unperturbed by the CuAAC reaction, demonstrating that the structure of the metallohelix architecture is preserved. The successful synthesis of all the “clicked” complexes was also confirmed by high resolution electrospray mass spectrometry (ESI Fig. S18–23†). For instance, a strong signal was observed by electrospray mass spectrometry at \(m/z\) 464.1580 Da for the tetracenotonic molecular ion of \(R_6\Delta_{\text{Fe}}\text{HHT-[Fe}_2\text{L}_3^{3+}]\text{Cl}_4\) within 0.001 Da of the calculated value for \(C_{105}H_{93}Fe_{21}N_{21}O_{6}\) (m/z 464.1582 Da). Inductively-Coupled Plasma Mass Spectrometry (ICP-MS) analysis of the iron triplex metallohelices revealed that only trace amounts of copper could be detected.

Subsequently fourteen new benzyl triazole-functionalised metallohelix enantiomers were isolated in a similar manner. Remarkably, the carboxylic acid derivatives \([\text{Fe}_2\text{L}_3^{3+}]^{+}\) were accessible, despite the stability of Fe carboxylates. In all cases, the triplex architecture was retained, even in the case of conventionally highly labile Zn(n). Characterizing data including NMR, MS, circular dichroism (CD), IR and microanalysis are detailed in ESI.† NMR and UV-vis experiments indicate that little decomposition of the product \([\text{Fe}_2\text{L}_3^{3+}]\text{Cl}_4\) occurred over months in aqueous solution (ESI Fig. S14, S15 and S17†).

Antiproliferative activity and cell line selectivity studies

The panel of Fe(n) compounds of Fig. 1 were initially evaluated alongside cisplatin for potency against the human epithelial colorectal cancer cell line HCT116 p53\(^{+/+}\) (wild-type p53) and non-cancerous human epithelial retinal pigment cells (ARPE-19) (Fig. 2). The modest potency of parent compound \([\text{Fe}_2\text{L}_3]^{3+}\) against HCT116 p53\(^{+/+}\) leads to a poor selectivity index: the ratio of IC\(_{50}\) values of ARPE-19 cells to HCT116 p53\(^{+/+}\) cells (see Fig. 2 – note log scale). While this is higher for the \(\Lambda\) enantiomer, the performance of the alkyne derivatives \([\text{Fe}_2\text{L}_3^{3+}]\text{Cl}_4\) is much improved, with potencies similar to that of cisplatin against HCT116 p53\(^{+/+}\) but giving rather better selectivity indices. The “clicked” metallohelices \([\text{Fe}_2\text{L}_3^{3+}]\text{Cl}_4\) perform better still. They are all more potent than cisplatin against the HCT116 p53\(^{+/+}\) colon cancer cells, with the exception of the more moderately active tricarboxylic acid \([\text{Fe}_2\text{L}_3^{3+}]\text{Cl}_4\). These complexes also exhibit enantioselectivity; in all cases the \(\Delta_{\text{Fe}}\) compounds are more active than \(\Delta_{\text{Fe}}\) [Fig. 2a]. Pleasingly, the new metallohelices are all significantly less toxic to non-cancerous ARPE-19 cells with the \(\Delta_{\text{Fe}}\) enantiomers being substantially less toxic than \(\Delta_{\text{Fe}}\) [Fig. 2b]. The resulting selectivity indices show that the \(\Delta_{\text{Fe}}\) enantiomers are all more selective than their \(\Delta_{\text{Fe}}\) analogues; both \([\text{Fe}_2\text{L}_3^{3+}]\text{Cl}_4\) and \([\text{Fe}_2\text{L}_3^{3+}]\text{Cl}_4\) have a selectivity indices.
for these particular cell lines of >30. On the basis that the former is the simpler compound, it was selected from the click derivatives for further study.

Accordingly, the compounds \( \Delta-[\text{Fe}_2\text{L}^1\text{Cl}_4] \), \( \Delta-[\text{Fe}_2\text{L}^2\text{Cl}_4] \) and \( \Delta-[\text{Fe}_1\text{L}^{1,3}\text{Cl}_4] \) were screened against a larger panel of cell lines of different tissue origins (colon, ovarian, cervical and breast...
The metallohelices were without exception more active against each of the seven cancer cell lines than the four different non-cancer cell lines. Furthermore, they display an overwhelmingly favourable selectivity compared with the clinically used chemotherapeutic agent cisplatin. In most instances the compound Δ-[Fe2L3a]Cl4 was the most selective.

We noted that while conversion of alkyne \( \Delta-[\text{Fe2L}^2_3]\text{Cl}_4 \) to benzyltriazole \( \Delta-[\text{Fe2L}^3_3]\text{Cl}_4 \) had little effect on potency in the HCT116 colon cancer cells, potency in the ovarian and breast cancer cell lines was increased \( \sim 7 \) to 25 fold (Table 1). In contrast, activity against three of the four non-cancer cell lines was only modestly increased (by \( <1.3 \) fold in HMF, \( \sim 1.5 \) fold in ARPE19 and \( \sim 2 \) fold in MRC-5 pd30). The click modification resulted in a \( \sim 4 \) fold increase in potency towards the cisplatin-resistant ovarian cancer cells (A2780cisR) compared to the cisplatin-sensitive parental cells indicating a lack of cross-resistance and, unsurprisingly, a distinct mechanism of action to that of the DNA “alkylator” cisplatin.

The p53 tumour suppressor gene is one of the most frequently mutated in cancer, commonly causing increased resistance to chemotherapeutic drugs. While accordingly here cisplatin was found to be \( >2 \) fold less active towards HCT116 p53\(^{-/-}\) cells than the p53\(^{+/+}\) isogenic clones (Table 1), such a loss of potency was not observed for any of the Δ-metallohelices.

### Mechanistic studies

We noted that the enantiomer potencies for \([\text{Fe2L}^2_3]\text{Cl}_4\) (i.e. \( \Delta > \Lambda \)) against cancer cells were reversed for \([\text{Fe2L}^3_3]\text{Cl}_4\) and the other click derivatives. We thus compared the effects of the enantiomer pairs on the cell cycle profile of asynchronously growing cells. Striking enantiomeric and structure-dependent differences were observed (Fig. 4) implying different mechanisms of action. Further, since the high selectivity index compound \( \Delta-[\text{Fe2L}^3_3]\text{Cl}_4 \) did not induce significant alteration in the cell cycle profile, the induction of cell death via apoptosis, a target of many anticancer drug treatments, was investigated. To our surprise, induction of apoptosis was not observed via a membrane phosphatidylserine (PS) assay\(^{27-29}\) for HCT116.

### Table 1

<table>
<thead>
<tr>
<th>Cell line</th>
<th>( \Delta-[\text{Fe2L}^2_3]\text{Cl}_4 )</th>
<th>( \Delta-[\text{Fe2L}^3_3]\text{Cl}_4 )</th>
<th>( \Delta-[\text{Fe2L}^3_3]\text{Cl}_4 )</th>
<th>Cisplatin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cancer cell lines</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCT116 p53(^{+/+}) (colon)</td>
<td>21.4 ± 1.4(^{b})</td>
<td>2.9 ± 0.9(^{b})</td>
<td>2.2 ± 1.0(^{b})</td>
<td>3.3 ± 0.4(^{b})</td>
</tr>
<tr>
<td>HCT116 p53(^{-/-}) (colon)</td>
<td>7.7 ± 3.2(^{b})</td>
<td>3.4 ± 0.2(^{b})</td>
<td>3.3 ± 0.3(^{b})</td>
<td>7.5 ± 0.7(^{b})</td>
</tr>
<tr>
<td>A2780 (ovarian)</td>
<td>6.38 ± 0.1</td>
<td>6.1 ± 0.8</td>
<td>0.9 ± 0.2</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td>A2780cisR (ovarian)</td>
<td>4.43 ± 0.1</td>
<td>6.1 ± 0.3</td>
<td>0.24 ± 0.02</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>HeLa (cervical)</td>
<td>3.8 ± 0.9</td>
<td>16 ± 6</td>
<td>7.6 ± 0.5</td>
<td>14.0 ± 0.9</td>
</tr>
<tr>
<td>MCF-7 (breast)</td>
<td>2.4 ± 0.4</td>
<td>16 ± 2</td>
<td>2.2 ± 0.2</td>
<td>12.9 ± 0.6</td>
</tr>
<tr>
<td>MDA-MB-231 (breast)</td>
<td>7 ± 1</td>
<td>22 ± 1</td>
<td>2.1 ± 0.2</td>
<td>22 ± 2</td>
</tr>
<tr>
<td><strong>Non-cancer cell lines</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ARPE-19 (retinal)</td>
<td>31 ± 12(^{b})</td>
<td>100 ± 5(^{b})</td>
<td>66 ± 7(^{b})</td>
<td>6.4 ± 1.0(^{b})</td>
</tr>
<tr>
<td>MRC-5 pd30 (lung)</td>
<td>31 ± 6</td>
<td>65 ± 5</td>
<td>32 ± 5</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>HMF (breast)</td>
<td>18 ± 3(^{b})</td>
<td>14 ± 2(^{b})</td>
<td>11 ± 1(^{b})</td>
<td>18 ± 2(^{b})</td>
</tr>
<tr>
<td>WI-38 (lung)</td>
<td>&gt;100(^{b})</td>
<td>&gt;100(^{b})</td>
<td>16 ± 3(^{b})</td>
<td>2.2 ± 0.8(^{b})</td>
</tr>
</tbody>
</table>

\(^{a}\) The experiments were performed in triplicate or quadruplicate. The cells were treated with the investigated compounds for 72 h, unless otherwise stated. The results are expressed as mean values ± SD from three or four independent experiments. \(^{b}\) Cells were treated for 96 h.
p53+/+ cells except after prolonged exposure at 2 × IC50 (ESI Fig. S26†).

Time-dependent cellular response profiles (TCRPs) produced by impedance-based monitoring provides predictive mechanistic information for the action of small molecules.32–33 Ovarian cancer cells (A2780) that have been treated with \( \Delta\text{-}[\text{Fe}_2\text{L}^1\text{Cl}_4] \), \( \Delta\text{-}[\text{Fe}_2\text{L}^2\text{Cl}_4] \) and \( \Delta\text{-}[\text{Fe}_2\text{L}^3\text{Cl}_4] \) show clearly distinct TCRPs (Fig. 5). For the parent metallohelix \( \Delta\text{-}[\text{Fe}_2\text{L}^1\text{Cl}_4] \), the initial rise in Cell Index (CI) impedance signal is less apparent than for other compounds and the period of signal elevation is the shortest. For the alkyne \( \Delta\text{-}[\text{Fe}_2\text{L}^2\text{Cl}_4] \) the CI signal increases to ca. 1.7× of the control and the peak is relatively broad, the signal decreasing steadily over the measurement period. For the benzyl triazole derivative \( \Delta\text{-}[\text{Fe}_2\text{L}^3\text{Cl}_4] \) the CI signals reach a much sharper dose-dependent maximum. A TCRP profile database search31 indicated a similarity with that for compounds that inhibit Na+/K+ stimulated ATPases; a highly conserved integral cell membrane pump expressed in virtually all cells of higher organisms that maintains ionic concentration gradients.34 An established rubidium-based assay35 subsequently showed that \( \Delta\text{-}[\text{Fe}_2\text{L}^3\text{Cl}_4] \) did indeed inhibit uptake of the cation in A2780 and HCT116 p53+/+ cell lines under these conditions, by 35–47% (Fig. 6). This performance is comparable with that of the known potent Na+/K+-ATPase inhibitor ouabain36 (39–57% inhibition). In contrast \( \Delta\text{-}[\text{Fe}_2\text{L}^1\text{Cl}_4] \) and \( \Delta\text{-}[\text{Fe}_2\text{L}^2\text{Cl}_4] \) had little if any effect.

**Antimetastatic properties of metallohelices**

Colorectal cancer is one of the four most common causes of cancer deaths,37 and in 90% of instances mortality is ascribed to
metastasis, for which there are currently no effective treatments. Ouabain has been reported to inhibit cancer cell migration and invasion and to possess antimetastatic activity through its action capable of forming colonospheres from single cells, and have been utilized in several studies as a CSC model. The HCT116 p53+/+ cells were treated with Δ-[Fe2L3]Cl4 and Δ-[Fe2L3]Cl4 at their respective IC30 concentrations for 72 h, and subsequently cultured as single cell suspensions in serum-free media. While both Δ-[Fe2L3]Cl4 and Δ-[Fe2L3]Cl4 were found to inhibit colonosphere formation in HCT116 p53+/+ under these conditions, Δ-[Fe2L3]Cl4 in particular was significantly more effective than the CSC-selective drug salinomycin (ESI Table S3 and Fig. S27, S28†). Subsequently, significant colonosphere inhibition by both Δ-[Fe2L3]Cl4 and

Cancer stem cell (CSC) targeting

CSCs represent a reservoir of resistant and highly aggressive cancer cells that can remain when the bulk of a tumour has been eradicated, leading to formation of secondary or tertiary tumours. The failure to eliminate or inhibit CSCs is thereby a major cause of failure of existing anti-cancer treatments and is a key challenge to improving patient outcomes.

The HCT116 p53+/+ cells possess a cancer stem cell fraction capable of forming colonospheres from single cells, and have been utilized in several studies as a CSC model. The HCT116 p53+/+ cells were treated with Δ-[Fe2L3]Cl4 and Δ-[Fe2L3]Cl4 at their respective IC30 concentrations for 72 h, and subsequently cultured as single cell suspensions in serum-free media. While both Δ-[Fe2L3]Cl4 and Δ-[Fe2L3]Cl4 were found to inhibit colonosphere formation in HCT116 p53+/+ under these conditions, Δ-[Fe2L3]Cl4 in particular was significantly more effective than the CSC-selective drug salinomycin (ESI Table S3 and Fig. S27, S28†).
D-[Fe₂L₃a]Cl₄ was also observed in CSC-enriched cells (HCT116.CD133+). The compounds were equally or slightly more effective than salinomycin in reduction in the number and average size of colonospheres [Fig. 9a–f].

Next, we compared the effects of D-[Fe₂L₁]Cl₄, D-[Fe₂L₃a]Cl₄ and salinomycin on HCT116.CD133⁺ and HCT116.CD133⁻ cells (Fig. 9 and ESI Fig. S30†) these data indicate that D-[Fe₂L₃a]Cl₄ and salinomycin effectively inhibit colonosphere formation from HCT116.CD133⁺ cells, but do not significantly inhibit colonosphere formation from HCT116.CD133⁻ cells. This result can be interpreted to mean that D-[Fe₂L₃a]Cl₄ and salinomycin are CSC-selective agents. On the other hand, D-[Fe₂L₃a]Cl₄ also effectively inhibits colonosphere formation from both HCT116.CD133⁺ and HCT116.CD133⁻ cells, although being less effective in CD133⁻ negative cells. The reasonable explanation of the latter result is that D-[Fe₂L₃a]Cl₄ exhibits a lower selectivity for CSCs than D-[Fe₂L₁]Cl₄ and salinomycin being able to effectively kill both differentiated cancer cells and CSCs. As effective cancer treatments must attack both rapidly-dividing differentiated (non-stem) cancer cells and CSCs, D-[Fe₂L₃a]Cl₄ appears to be a promising candidate compound able to overcome limitations connected with the use of a number of conventional chemotherapeutics.

The data in Fig. 9g arise from a clonogenic assay that examines the capability of a single cell to grow into a large colony through clonal expansion.⁷⁷ CSC-enriched HCT116.CD133⁻ cells incubated for 48 h with 30 µM D-
Fig. 9 Growth inhibitory effects in HCT116.CD133+ cancer stem cells. Representative microscopy images of the HCT116.CD133+ colonospheres in the absence (a) and presence of salinomycin (b), Δ-[Fe₂L³]Cl₄ (c), and Δ-[Fe₂L⁵a]Cl₄ (d), treated at their respective IC₅₀ values for 6 days (scale bar: 100 μm). Quantification of colonosphere formation (e and f) under the same conditions. Clonogenic assay on the HCT116.CD133+ (g) showing the colony forming efficiency (i.e. the number of colonies that formed post-drug treatment, with respect to the number of cells seeded) after treatment with different concentrations of salinomycin, (grey circles), Δ-[Fe₂L³]Cl₄ (black open circle), and Δ-[Fe₂L⁵a]Cl₄ (black squares) for 48 h, following growth for 8 days. Data represent the mean value and SD from three independent experiments. p < 0.01, versus control.

[Fe₂L³a]Cl₄, exhibited no surviving cells after being allowed to grow for 8 d; an effect comparable to that of conventional salinomycin, Δ-[Fe₂L¹]Cl₄ was less effective.

The selective anti-cancer stem cell effects of Δ-[Fe₂L³a]Cl₄ were further demonstrated (ESI Table S4†). The IC₅₀ of 1.21 ± 0.25 μM in CSC-enriched HCT116.CD133+ is around half in HCT116 p53⁺ cells under the same conditions; a better differential than that observed with salinomycin.

The studies aimed at the mechanism of action of the investigated metallohelices on CSCs is in progress in our laboratories and will be published in a separate article. Nevertheless, we demonstrate in our manuscript that Δ-[Fe₂L¹]Cl₄ and Δ-[Fe₂L³a]Cl₄ were equally or slightly more effective in killing CSCs than CSC-selective salinomycin (ESI Fig. S30†). It was shown recently that nucleolin is likely a salinomycin-binding target and a critical regulator involved in human neuroblastoma CSC activity. It is, therefore, possible, due to the similar effectivity of the investigated metallohelices (Δ-[Fe₂L¹]Cl₄ and Δ-[Fe₂L³a]Cl₄) and salinomycin to kill HCT116.CD133+ CSCs, that their binding to nucleolin may also be responsible for the anticancer and anti-CSC like cell activities of Δ-[Fe₂L¹]Cl₄ and Δ-[Fe₂L³a]Cl₄.

Conclusions

One of the key advantages of our metallohelix assemblies is their great stability with respect to dissociation or hydrolysis. This sort of stability will be necessary in order for any such compound to find its way into clinical use, and has here allowed the use of an extremely efficient CuAAC post-assembly modification of the triplex alkylnyl enantiomers [M₄L²]. This reaction gave rapid access to a new range of functionalised compounds and led to the discovery of Δ-[Fe₂L⁵a]Cl₄; a compound with an unusual combination of pharmacological properties.

In a study involving an unusually wide range of cancer and non-cancer cell types, the new “clicked” compounds demonstrated enhanced potency. More importantly however the selectivity for cancerous over non-cancerous cells was greatly improved; this bodes well for the development of compound with a wider therapeutic window than conventional chemotherapeutic agents such as cisplatin. Further, in contrast to observations for conventional drugs, a p53 mutated cell line did not show resistance to the compound.

While some part of the observed selectivity of Δ-[Fe₂L³a]Cl₄ probably arises from electrostatic targeting of the anionic outer leaflet of cancer cells (as is proposed for cationic anticancer peptides) the enantiomer effects observed here point to greater subtlety. The Δ compound is much more selective than Δ for cancer cells, and this is reflected in a remarkable difference in cell cycle arrest observations. It was also discovered that Δ-[Fe₂L³a]Cl₄ inhibits Na⁺/K⁺ ATPase activity with potency comparable to that of the conventional inhibitor ouabain.

These mechanistic observations led to the discovery of a remarkable array of properties of Δ-[Fe₂L⁵a]Cl₄ alongside its high potency and selectivity. The compound suppresses key metastatic capacities of cancer cells, reducing their ability to detach from other cells, migrate, invade and re-adhere elsewhere. Compound Δ-[Fe₂L⁵a]Cl₄ exhibits selective toxicity for colon cancer stem cell-enriched cell populations, challenging some of the most selective compounds of any kind identified to date. Additionally, Δ-[Fe₂L⁵a]Cl₄ inhibits the formation of colonospheres by specifically targeting CD133-positive, CSC-like...
cells. Thus $\Delta[\text{Fe}_2\text{L}_3\text{Cl}_4]$ is identified as a potential lead compound for investigation as a selective anti-cancer, anti-metastatic and CSC-targeting drug.

More generally, while the precise molecular basis for the difference between the benzyltriazolyl-clicked compounds and the parent enantiomers is not known, it is clear that these discoveries would be much harder to unearth without access to post-assembly modification. Further, we now know that metallohelices, like conventional medicinal compounds, respond to chemical modification in such a manner as to facilitate optimization of biological and physicochemical properties.

**Experimental**

Full details of synthesis, characterization and anti-cancer experiments are provided in the electronic ESI.† Outlines of key procedures are detailed in the following.

**Synthesis of water-soluble alkyne-functionalised triplex metallohelices**

Anhydrous iron(II) chloride (2 equiv.) was added to a stirred solution of the desired chiral amine, 2-[2′,2′-bipyridin]-5-ylmethoxy)-1-phenylethan-1-amine (3 equiv.) and 5-(prop-2-yn-1-yl)picolinaldehyde (3 equiv.) in methanol (20 ml) at ambient temperature to give a purple solution that was then heated to reflux (85 °C) for 48 h. The mixture was allowed to cool to ambient temperature, filtered through a Celite plug, and the solvents were removed in vacuo to give HHT-[Fe$_2$L$_3$Cl$_4$] as a dark purple solid (>95% yield).

**Post-assembly modification of triplex metallohelices**

HHT-[Fe$_2$L$_3$Cl$_4$] (1 equiv.) and the chosen benzyl azide (4.5 equiv.) were dissolved in methanol (10 ml) in the presence of copper(II) iodide (0.1 equiv.). The reaction mixture was heated at 65 °C for 18 h under inert argon atmosphere. After cooling to ambient temperature, the suspension was filtered to remove copper salts and the purple product HHT-[Fe$_2$L$_3$Cl$_4$] was isolated by the addition of ethyl acetate. NMR, infrared and high-resolution mass spectrometric data were consistent with the proposed formulations. CD spectra of enantiomers in methanol were equal and opposite.

**Antiproliferative activity (MTT assay)**

The human ovarian carcinoma cisplatin-sensitive A2780 cells, cisplatin-resistant A2780cisR (a cisplatin-resistant variant of A2780 cells), human cervical carcinoma HeLa cells, human breast cancer MCF-7 cells and human colorectal carcinoma cells HCT-116 (for experiments performed at the Czech Academy of Sciences) were kindly supplied by Professor B. Keppler, University of Vienna (Austria). Human colon carcinoma cells expressing p53 (HCT116 p53$^{+/+}$) were a kind gift of Dr M. Brazdova, Institute of Biophysics, Brno (Czech Republic). Highly invasive breast carcinoma MDA-MB-231 cells and human MRC-5 pd30 cells derived from normal lung tissue were purchased from the European collection of authenticated cell cultures (ECACC) (Salisbury, UK). Isogenic clones of p53$^{+/+}$ and p53$^{-/-}$ HCT116 colon carcinoma cells for experiments performed at the University of Huddersfield were a kind gift from Bert Vogelstein (Johns Hopkins University, Maryland, USA). ARPE19 and WI38 cells were both purchased from ATCC (American Type Culture Collection) and HMF cells were purchased from ScienCell Research Laboratories, Inc.

Cells were incubated in 96-well plates in complete medium, containing DMEM supplemented with 10% fetal calf serum and l-glutamine (2 mM). Plates were incubated for 24 h at 37 °C in an atmosphere of 5% CO$_2$ before drug exposure, then incubated for 96 h with drug. A volume of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (0.5 mg ml$^{-1}$) was added to each well and incubated for a further 4 h. These solutions were removed, dimethyl sulfoxide was added to each well, and the absorbance at 540 nm was read using a Thermo Scientific Multiskan EX microplate photometer. The IC$_{50}$ values were determined from a plot of percentage cell survival against drug concentration (in μM). All assays were conducted in triplicate and the mean IC$_{50}$ ± standard deviation was determined.

**Cell cycle assay**

PBS (300 μL) containing propidium iodide (50 μg ml$^{-1}$) and RNase A (80 μg ml$^{-1}$) was added to drug-treated cells before incubation for 30 min and FACS analysis. The assay was repeated four times with each compound and the mean percentage of cells in each phase ± standard deviation was determined. Red fluorescence was observed at 488 nm excitation by flow cytometry and data were analysed using Flowjo V10.

**Time-dependent cellular response profiling**

The impedance monitoring of cell growth was performed using an xCELLigence RTCA SP Real-time cell analyser. Cells were added and grown for 22–28 h, before tested compounds were added to the medium at varying concentrations. The impedance was measured for an additional 80 h.

**Rubidium-based assay**

Cells were seeded in 6-well plates and incubated for 24 h at 37 °C in an atmosphere of 5% CO$_2$. Cells were then incubated with drug for 6 h, and subsequently the medium was removed and cells were washed with PBS. Cells were then incubated with RbCl (5.4 mM) for 3 h, washed and counted. Rubidium content was determined by ICP-MS.

**Wound healing assay**

Cells were seeded in 12-well plates and incubated in complete medium (10% FBS/DMEM/gentamycin) at 37 °C in an atmosphere of 5% CO$_2$. The medium was removed and the bottom of the well was scratched with a 10 μL pipette tip (550–650 μm gaps). Wells were washed to remove detached cells before drug was added, dissolved in complete or starving medium, and plates were incubated for 24 h. Images were taken at several time intervals post scratching, and automated analysis was performed using TScratch software (MATLAB).
Inhibition of colonosphere formation

Cells were treated with drug (IC_{30} concentration) for 72 h, washed, harvested with StemPro Accutase, and plated in ultra-low attachment 96-well culture plates (300 cells per well). Cells were cultured in for 6 days without disturbing the plates or replenishing the medium and the number/size of spheres were determined using an inverted microscope.

Clonogenic assays

Cells were seeded in ultra-low attachment 6-well plates and cultured for 4 days, to allow pre-spheroid formation. Cells were treated with drug for 48 h, and dissociated into single cell suspensions using StemPro Accutase. Single cells were seeded in ultra-low attachment 96-well culture plates (300 cells per well). Cells were cultured in for 6 days without disturbing the plates or replenishing the medium and the number/size of spheres were determined using an inverted microscope.

Conflicts of interest

There are no conflicts to declare.

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Notes and references
