Derivatisation of buforin IIb, a cationic henicosapeptide, to afford its complexation to platinum(II) resulting in a novel platinum(II)-buforin IIb conjugate with anti-cancer activity

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Herein we report the synthesis of buforin IIb, its novel malonate derivative malBuf and its Pt(II) complex cis-[Pt(NH$_3$)$_2$(malBuf)$_2$]. We decided to harness the cell targeting, cell-penetrating and anti-proliferative effects of buforin IIb to help target a cytotoxic dose of a Pt DNA binding species, {Pt(NH$_3$)$_2$} to cancer cells whilst also delivering a peptide with potent anti-cancer properties. Preliminary in vitro data shows cis-[Pt(NH$_3$)$_2$(malBuf)$_2$] to be more cytotoxic against the cisplatin resistant ovarian cancer cell line (A2780 CisR) relative to buforin IIb, cisplatin and cis-[Pt(NH$_3$)$_2$(malBuf)$_2$].

Cancer remains a leading cause of death worldwide. Conventional cancer therapies, while highly efficacious, are generally non-specific for cancer cells over healthy cells resulting in dose-limiting toxic side effects. Drug resistance is also a major drawback associated with many therapies. Of the chemotherapeutics in clinical use, platinum drugs, namely cisplatin, carboplatin and oxaliplatin, are amongst the most widely employed. Pt drugs exert their cytotoxic effect by binding irreversibly to DNA nucleobases leading ultimately to induction of apoptosis.

Peptides have emerged as promising vectors for the selective delivery of cytotoxic Pt payloads to tumour cells. The conjugation of Pt to peptides however can be associated with significant synthetic challenges. The classical synthetic scales of these two classes of molecules differ by at least one order of magnitude and the synthetic requirements of the two partners and of the linker cannot sometimes be reconciled. The fact that many peptide amino acid side chains carry Pt reactive nitrogen or sulfur nucleophiles which strongly and irreversibly bind to Pt is also an issue, potentially preventing the Pt from ultimately binding with its target DNA. The earliest report of a Pt-peptide conjugate dates back to 1996 when Kelland et al. conjugated derivatives of the minor groove binding agents netropsin and distamycin to Pt(II). Further developments in Pt-peptide research were no doubt facilitated with advances in synthetic strategies compatible with solid-phase peptide synthesis (SPPS), with the first solid phase synthesis of a Pt-peptide conjugate reported in 2000. However, it should be noted that SPPS usually requires strongly acidic conditions and most often a cocktail of highly nucleophilic scavengers to sever the peptide-resin anchor without modifying the peptide. Such conditions are not always compatible with Pt chemistry. Of the Pt(II) peptide derivatives subsequently reported, most link the Pt to the peptide via a N,N'-linker system. The peptide thus remains tightly bound to the Pt metal centre under cellular conditions. To the best of our knowledge, there are only a handful of examples of Pt(II)-peptide conjugates in which the peptide is bound to the Pt via an O,O'-linker system. Examples include Pt-cyclic peptide conjugates that target the CD13 receptor over-expressed on cancer cells. Another example includes a Pt conjugated to a mitochondrial targeting decapeptide. The Pt(II)-peptide conjugates to date focus mainly on exploiting the peptide as a ‘homing device’ for the targeted delivery of a cytotoxic Pt(II) payload to tumour cells. We previously reported a Pt(II)-peptide conjugate in which the peptide, P18, served to not only selectively deliver its Pt payload but also possessed intrinsic anti-cancer properties. The P18 peptide coordinated the Pt via an ethylenediamine N,N'-linker. Employing an O,O'-linker system in our opinion may have an advantage over N,N'-linkers if the peptide itself has anti-cancer properties. The peptide should selectively deliver the complex to the tumour cells where, upon tumour cell entry, one might hypothesise that the Pt-peptide conjugate would be activated in much the same way as carboplatin, releasing concurrently the Pt-DNA binding moiety and the peptide free to interact with its cellular target.

Buforin IIb (1) is a cell-penetrating cationic anti-microbial peptide (AMP) consisting of 21 amino acids which possesses not only potent anti-microbial activity but also potent anti-proliferative activity, Fig. 1. When screened against the National Cancer Institute’s 60 cancer cell lines, Buforin IIb was found to be cytotoxic against all cell lines with IC$_{50}$ values ranging from 7.2mg/ml against lung HOP-92 to 23.9 mg/ml against breast T47-D cell lines. Buforin IIb has also demonstrated significant tumour suppression activity in vivo. It has also been found to be selective for tumour cells over normal cells based on its electrostatic interactions with cell surface negatively charged gangliosides. Unlike some AMPs, Buforin IIb transverses the membrane without compromising its integrity and acts intracellularly where it induces apoptosis via a mitochondria-dependent pathway. The endoplasmic reticulum has also recently been cited as a potential target of buforin IIb.
We decided to harness the cell targeting, cell-penetrating and anti-proliferative effects of buforin IIb to help target a cytotoxic dose of a Pt DNA binding species, [Pt(NH$_3$)$_2$] to cancer cells whilst also delivering a peptide with potent anti-cancer properties. Buforin IIb was also chosen on the basis that the peptide should be compatible with Pt chemistry; the side chains of buforin IIb’s amino acid sequence are mostly non-reactive with Pt under the conditions required to complex an O,O’-bidentate linker with cis-[Pt(NH$_3$)$_2$(OH$_2$)$_2$] to thus providing an opportunity to construct a cancer cell selective Pt-buforin IIb conjugate. We chose to synthesize the unnatural D stereoisomer of buforin IIb; the D-stereoisomeric form should possess inherent resistance to proteolytic degradation potentiating its in vivo biostability and anti-proliferative activity.""""12 We then chose to incorporate a malonate-type linker to generate malBuf (2) to facilitate its binding to Pt generating cis-[Pt(NH$_3$)$_2$(malBuf)$_2$] (3), Fig. 2. Because Pt drugs react indiscriminately in the body giving rise to many of their drawbacks, we speculated that the presence of the peptide, with its known affinity for tumour cells, might also confer selectivity to the drug candidate thereby reducing the non-selective toxicity of classical Pt drugs as well as potentially offering an advantage over concurrent administration of classical Pt drugs with peptides.

We envisaged that cis-[Pt(NH$_3$)$_2$(malBuf)$_2$] would undergo selective cellular accumulation, entering the cells via a distinct pathway to classical Pt drugs and, once inside the cell, would be activated in much the same way as carboplatin releasing two cytotoxic payloads; the (Pt(NH$_3$)$_2$) moiety free to bind DNA nucleobases and the cytotoxic peptide, both capable of inducing cell death but via different mechanisms.

Herein we describe the first example of a cytotoxic AMP tethered to Pt via an O,O’-bidentate linker; thus combining two drug entities into one drug molecule. The synthesis of the derivatized buforin (malBuf), its complexation to cisplatin to afford cis-[Pt(NH$_3$)$_2$(malBuf)$_2$] and preliminary cytotoxicity studies are reported. cis-[Pt(NH$_3$)$_2$(malBuf)$_2$] incorporates a peptide consisting of 21 amino acids and, as such, also represents to the best of our knowledge, the largest peptide to date conjugated to Pt.[II]

![Chemical structures of malBuf (2) and cis-[Pt(NH$_3$)$_2$(malBuf)$_2$] (3)](image)

Automated SPPS using a standard fluorenlymethoxycarbonyl/tert-butyl (Fmoc/tBu) protection strategy provided a facile avenue for the generation of buforin IIb, albeit in small scale, as previously reported in the literature.11 Attachment of the protected malonate-linker to the resin-bound buforin IIb was then performed manually, using HATU/DIEA coupling chemistry. The malonate-modified peptide was finally cleaved from the resin, lyophilized and purified by reverse-phase HPLC. It was characterised by HPLC, Fig. 3 (ESI) and MS (Fig. 4 (ESI)) analysis. Reaction of cis-[Pt(NH$_3$)$_2$(OH$_2$)$_2$] with malBuf at pH ~6.6 yielded our desired product cis-[Pt(NH$_3$)$_2$(malBuf)$_2$]. Any excess Pt starting material was removed using gel filtration on a Sephadex-G10 column. An iodine test was performed to identify peptide in the collected fractions which were combined, frozen and lyophilized. cis-[Pt(NH$_3$)$_2$(malBuf)$_2$] may be found in the ESI.

![ESI-MS of cis-[Pt(NH$_3$)$_2$(malBuf)$_2$]](image)

Table 1 Anti-proliferative effect of test agents against A2780 and A2780 cisR ovarian cancer cell lines A2780P and A2780cisR respectively and the non-tumorigenic, normal human dermal fibroblast cells, NHDF. Dose-response curves were used to calculate IC$_{50}$ values (Table 1). We previously determined the cytotoxicity of cis-[Pt(NH$_3$)$_2$(malonate)] against these cell lines and this data has also been included on the basis that cis-[Pt(NH$_3$)$_2$(malonate)] is a more appropriate reference standard than cisplatin because of the structural similarities in the Pt coordination environments of cis-[Pt(NH$_3$)$_2$(malonate)] and cis-[Pt(NH$_3$)$_2$(malBuf)$_2$].

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<th>Compound</th>
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<tr>
<td></td>
<td>A2780P</td>
</tr>
<tr>
<td></td>
<td>A2780cisR</td>
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<tr>
<td>cis-[Pt(NH$_3$)$_2$(malonate)]$^{13}$</td>
<td>13.0 ± 0.1</td>
</tr>
<tr>
<td>Buforin IIb</td>
<td>12.3 ± 0.8</td>
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<tr>
<td>malBuf</td>
<td>24.4 ± 3.2</td>
</tr>
<tr>
<td>cis-[Pt(NH$_3$)$_2$(malBuf)$_2$]</td>
<td>12.2 ± 1.0</td>
</tr>
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</table>

The novel cis-[Pt(NH$_3$)$_2$(malBuf)$_2$] was found to exhibit high toxicity towards both cisplatin sensitive and resistant cancerous cell lines A2780 and A2780 cisR with IC$_{50}$ values of 12.2 and 7.8 µM, respectively. The in vitro data shows no improvement in anti-

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**Table 1** Anti-proliferative effect of test agents against A2780 and A2780 cisR ovarian cell lines and NHDF cells following 72 hr continuous incubation with compound in the range 0 - 100 µM. Results presented are representative of three independent experiments (Mean ± S.E.M., n=3).

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**Fig. 2** Chemical structures of malBuf (2) and cis-[Pt(NH$_3$)$_2$(malBuf)$_2$] (3)
proliferative effect for cis-[Pt(NH$_3$)$_2$(malBuf$_2$H$_2$)] relative to butorfin Ilb for the A2780 parental cell line (IC$_{50}$ 12.2 vs. 12.3 µM) but perhaps most significant is its improved anti-proliferative effect on the cisplatin resistant variant relative to butorfin Ilb (IC$_{50}$ 7.8 vs. 12.7 µM). An increase in cellular cytotoxicity was also observed for the Pt-malbuforin Ilb conjugate towards the cisplatin resistant variant relative to cisplatin alone (IC$_{50}$ 7.8 vs. 9.7 µM). Interestingly, cis-[Pt(NH$_3$)$_2$(malBuf$_2$H$_2$)] has an order of magnitude greater cytotoxicity when compared to cis-[Pt(NH$_3$)$_2$(malonate)] against this A2780cisR variant. The only difference between cis-[Pt(NH$_3$)$_2$(malonate)] and cis-[Pt(NH$_3$)$_2$(malBuf$_2$H$_2$)] is the presence of the peptide in the latter complex – the significantly enhanced cytotoxicity observed in this resistant variant must thus be related to the peptide suggesting that the presence of the peptide in this complex is contributing to circumvention of some resistant factors.

We employed the D stereoisomeric form of buforin Ilb; aside from the achiral glycine, all other amino acids used for its synthesis were in the D configuration. We anticipated that this would prolong the intracellular survival and biostability of buforin Ilb by evading proteolytic degradation and increase cell cytotoxicity. We therefore expected to observe enhanced cytotoxicity of our Pt-peptide conjugate over each agent alone but this was not found to be the case. The malBuforin Ilb was the least cytotoxic, mostly due to conjugate over each agent alone but this was not found to be the case. The malBuforin Ilb was the least cytotoxic, mostly due to conju

Conclusions

In conclusion, we have described a derivatisation of the cationic AMP buforin Ilb to facilitate its complexation to Pt(II) resulting in a novel Pt-buforin Ilb conjugate with cytotoxic activity. The complex cis-[Pt(NH$_3$)$_2$(malBuf$_2$H$_2$)] exhibits an order of magnitude greater cytotoxicity against the cisplatin resistant A2780 variant relative to treatment with the cis-[Pt(NH$_3$)$_2$(malonate)] control indicating the potential of utilizing a multi-modal approach such as the one described to overcome resistance. This was a proof of concept study investigating not only the feasibility of linking an anti-cancer active and cancer cell targeting peptide to Pt(II) but also one in which the peptide consisted of a sequence in excess of 20 amino acids. As buforin Ilb has an amino acid content representative of AMPs, enriched with cationic amino acids such as arginine and hydrophobic residues including branched amino acids, the approach described herein could be extended to other sequences of this class of peptides. This therefore presents a potential new therapeutic avenue for further investigation.

Acknowledgements

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Notes and references

† Full experimental detail, together with HPLC traces of buforin Ilb and malBuforin Ilb and MS of buforin Ilb, malBuf and cis-[Pt(NH$_3$)$_2$(malBuf$_2$H$_2$)] may be found in the ESI.