Platinum(iv) oxaliplatin-peptide conjugates targeting memHsp70+ phenotype in colorectal cancer cells.

AUTHOR(S)

Aoife M. McKeon, Janis Noonan, Marc Devocelle, Brona M. Murphy, Darren M. Griffith

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A. M. McKeon,[a] J. Noonan,[b] M. Devocelle,[a] B. M. Murphy,[b] D. M. Griffith[a,*]

Novel Pt(IV) tumour penetrating peptide (TPP) conjugates are reported. They are the first example of metallodrugs to target a membrane bound heat shock protein 70 positive (memHSP70+) phenotype in cancer cells. The conjugates exhibit superior cytotoxicity as compared to oxaliplatin alone in Pt resistant colorectal cancer cells with relatively high memHSP70+ expression. Substiution of TPP in Pt(IV) peptide conjugates with scrambled peptide (ScP) essentially abolishes the observed cytoxicity.

The targeted delivery and uptake of anticancer drugs represent major challenges. There is currently considerable interest in the use of biocompatible peptides to endow clinically used drugs with superior selectivity for cancer cells and enhanced tumour cellular uptake.[1, 2] Peptides are increasingly easier and cheaper to produce and also have (i) better biodistribution, (ii) superior ability to penetrate tissues and (iii) are generally more stable than antibodies.[3]

Significantly therapeutic peptides have been clinically used in the treatment of multiple myeloma, prostate and breast cancer amongst others[4] and used in nuclear oncology, where for example the important PET tracer, ^{68}Ga labelled DOTA-D-Phe-Tyr-octreotide, targets the somatostatin receptors on the surface of neuroendocrine tumours.[5]

Many important recognition structures for imaging and targeting of cancer cells have been identified and reported in the literature. HSP70 for example is a stress-inducible chaperone, which maintains protein homeostasis during normal cell growth but during a stress response is upregulated and stabilises it's protein substrates until adverse conditions improve.[6] It is overexpressed in many cancers including colorectal cancer and is associated with cancer progression and poor prognosis.[7, 8]

More recently the selective expression of HSP70 on the plasma membrane (memHSP70) of cultured cancer cells including colorectal cancer cells was reported. Significantly memHSP70 was found to be present on 50% of tumours in a 1,000 patient cohort, though not expressed in healthy tissues.[3]

Multhoff and coworkers demonstrated that a mouse monoclonal antibody, cmHSP70.1, can be taken up by memHSP70+ cancer cells via endosomal pathways, and used the antibody to image memHSP70+ tumors *in vivo.*[3]

In addition they subsequently reported the specific binding of and rapid internalization of an HSP70-derived 14-mer peptide (tumour penetrating peptide, TPP, TKDNNLLGRFELSG), by memHSP70+ tumour cells. TPP matches an epitope within the oligomerization domain of the HSP70 molecule (aa 450–463).[3] TPP therefore has significant potential for the targeting of the large proportion of tumours which express memHSP70.

Platinum (Pt) compounds such as cisplatin, carboplatin and oxaliplatin have played a very important and well documented role in treating cancer.[9, 10] The cytotoxicity of Pt drugs, which enter cells and hydrolyse is linked to multiple mechanisms,[11] including for example effects in the cytoplasm such as oxidative and reticular stress and mitochondrial DNA damage but primarily and traditionally their ability to form nuclear DNA adducts. These events can lead to DNA damage responses, senescence and ultimately programmed cell death, apoptosis.[9-11] Significantly Lippard and coworkers have recently demonstrated that oxaliplatin, in contrast to cisplatin and carboplatin, does not kill cells via the DNA-damage response but by inducing ribosome biogenesis stress.[12]

Though Pt drugs are associated with high rates of clinical responses, they have noteworthy side effects due to indiscriminate toxicity. Many cancers including colorectal are also intrinsically resistant or acquire resistance to Pt-based therapies and in part due to reduced uptake.[11]

Attention in medicinal inorganic research is now being turned towards the development of metallodrugs, in particular Pt(IV) prodrugs, which contain bioactive ligands.[13]

[a] *Department of Pharmaceutical & Medicinal Chemistry, Royal College of Surgeons in Ireland, 123 St. Stephens Green, Dublin 2, Ireland.*
[b] *Department of Physiology & Medical Physics, Royal College of Surgeons in Ireland, 31A York Street, D2, Ireland.*

† Footnotes relating to the title and/or authors should appear here.
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Pt(IV) prodrugs are believed to be activated by reduction in the hypoxic and reducing intracellular milieu of cancer cells liberating the corresponding cytotoxic Pt(II) metabolite and two axial ligands.[13, 14]

In bifunctional Pt(IV) prodrugs a bioligand is often coordinated to a Pt(IV) centre by a carboxylate functionality. These ligands generally play a role (i) in drug targeting and delivery or (ii) possess inherent anticancer properties.[13, 15-21]

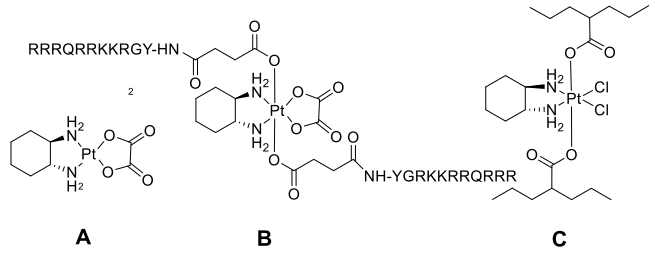

Figure 1 Structures of Oxaliplatin A, Pt(IV) cell penetrating peptide (TAT) conjugate B and *cis,cis,trans*-dichlorido(*trans*-(1*R*,2*R*)diaminocyclohexane)valproatoplatinum(IV) C.

Keppler and coworkers developed an oxaliplatin Pt(IV) TAT-(YGRKKRRQRRR) cell penetrating peptide conjugate[19] and Gibson *et al.* a Pt(IV) prodrug conjugate of the histone deacetylase inhibitor, valproic acid (VPA),[15] for example, Fig. 1.

We report the development of novel Pt(IV) oxaliplatin conjugates of the 14-mer tumour penetrating peptide, **TPP**, TKDNNLLGRFELSG, with a view to targeting a memHSP70+ phenotype in colorectal cancer cells.

TPP and the corresponding scrambled peptide, LNLETRLGFGDNKS (**ScP),** Figure 2, were assembled on a 0.5 mmol and 0.1 mmol scale respectively using automated solid phase peptide synthesis (SPPS), a standard Fmoc/tBu protection strategy, a Rink amide MBHA resin and DIC/oxyma pure coupling chemistry.[22]

As required, **TPP** was cleaved from the resin (90% TFA, 5% Phenol, 5% Water), lyophilized and purified by semi-preparative RP-HPLC and characterised by analytical RP-HPLC, Fig. S1 (ESI†) and HR-MS analysis, Fig. S6 (ESI†). For **TPP** purification of > 98 % was achieved and mass peaks at 1561.71 a.m.u (M^+) and 782.05 a.m.u. $(M+2H^+)^{2+}$ observed.

Platinum peptide conjugates were synthesised manually upon activation of the two free succinato carboxylic acid groups on the previously reported *cis,cis,trans*-dichlorido(*trans*-(1*R*,2*R*)diaminocyclohexane)oxalatodisuccinatoplatinum(IV) complex, *cis,cis,trans*-[Pt(1*R*,2*R*-DACH)(ox)(suc)$_2$], [19] with CDI and subsequent coupling in anhydrous DMF with the deprotected N-terminal threonine of the resin bound **TPP** sequence or N-terminal leucine of the resin bound **ScP** sequence. The final products were cleaved from the resin (90% TFA, 5% Phenol, 5% Water), lyophilized and purified by semi-preparative RP- HPLC.

For **TPP** and **ScP**, both reactions afforded, albeit in typical low yield,[19, 20] the corresponding Pt(IV) mono- and diconjugate peptide species; [Pt(1*R*,2*R*-DACH)(ox)(suc)(suc**TPP**)] **1**, [Pt(1*R*,2*R*-DACH)(ox)(suc**TPP**)$_2$] **2**, [Pt(1*R*,2*R*-DACH)(ox)(suc)(suc**ScP**)] **3** and [Pt(1*R*,2*R*-DACH)(ox)(suc**ScP**)$_2$] **4**, Fig. 2. The purity of the conjugates was confirmed by analytical RP-HPLC, Fig. S2-S5 (ESI†) and unambiguously characterised by HR-MS, Fig. S8-S14 (ESI†).

For Pt monoconjugates, **1** and **3**, purification of > 97 % and > 98 % respectively was achieved Fig. S2 and S4 (ESI†). The experimental mass spectral data are in good agreement with the predicted values and exhibit the expected isotopic mass distribution patterns. For **1** (ESI-MS) mass peaks at 1088.91 $(M+2H^+)^{2+}$ and 733.29 $(M+3H^+)^{3+}$ (Fig. S8) and for **3 at** 1088.96 $(M+2H^+)^{2+}$ (Fig. S13) were observed.

For Pt diconjugates, **2** and **4** purification of > 90 % and > 95 % were achieved respectively where an additional 8.01 % and 3.28 % of the samples respectively are attributed to the corresponding Pt monoconjugates, Fig. S3 and S5 (ESI†). For example in regards to the mass spectrum of **2,** Figure S10 (ESI), a mass peak at 1088.96 is clearly evident and corresponds with the m/z value for the corresponding Pt monoconjugate $(M+2H^+)^{2+}$.

Regarding ESI-MS for **2** mass peaks at 1240.52 $(M+2H^+)^{2+}$, 931.16 $(M+3H^+)^{3+}$ and for **4** mass peaks at 1240.55 $(M+2H^+)^{2+,}$ and 930.91 $(M+3H^+)^{3+}$ were observed.

In addition signals associated with the purely organic succinate-**TPP** were observed. For example for **1** and **2** using MALDI TOF mass peaks at 1662.80 and 1662.85 a.m.u. respectively are evident, (Fig. S11 and S12).

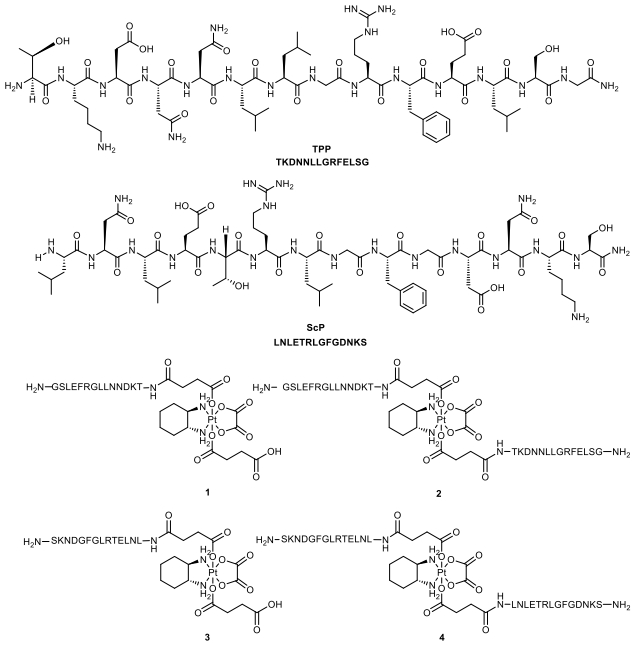

Figure 2 Structures of **TPP**, **ScP** and corresponding Pt mono- (**1** & **2**) and diconjugates (**3** & **4**).

In vitro cytotoxicity

A panel of four colorectal cancer cell lines, HT29, LoVo, HCT116 and HCT116 p53 -/- were selected to investigate the *in vitro* effects of cisplatin and oxaliplatin. As expected oxaliplatin the Pt drug of choice for the treatment of stage IV colorectal cancer, exhibits superior cytotoxicity to cisplatin in all four colorectal cancer cell lines examined. In addition oxaliplatin is more cytotoxic against the LoVo and HCT116 cell lines (relatively sensitive) as compared to the HT29 and HCT116 p53-/- cell lines (relatively resistant).

Table 1: IC50 (μM) values calculated for cisplatin and oxaliplatin against colorectal cell lines following 72 h of treatment (n=3).

	HT29	LoVo	HCT116	HCT116 p53 -/-
Cisplatin	19	5	6	14
Oxaliplatin	9[8]	0.5[8]	0.3[8]	5

HSP70 and memHSP70 expression

Western Blot analysis of whole cell lysates of HT29, LoVo, HCT116 and HCT116 p53 -/- colorectal cancer cell lines using a monoclonal HSP70 antibody showed positive HSP70 staining in all four colorectal cancer cell lines, in particular the HT29 cell line. In direct contrast the expression of HSP70 was scarcely observed for the normal human dermal fibroblast (NHDF) cell line, Fig. 3 A. This observation complements literature reports where HSP70 is observed to be overexpressed in cancer cells as opposed to normal cells.[7]

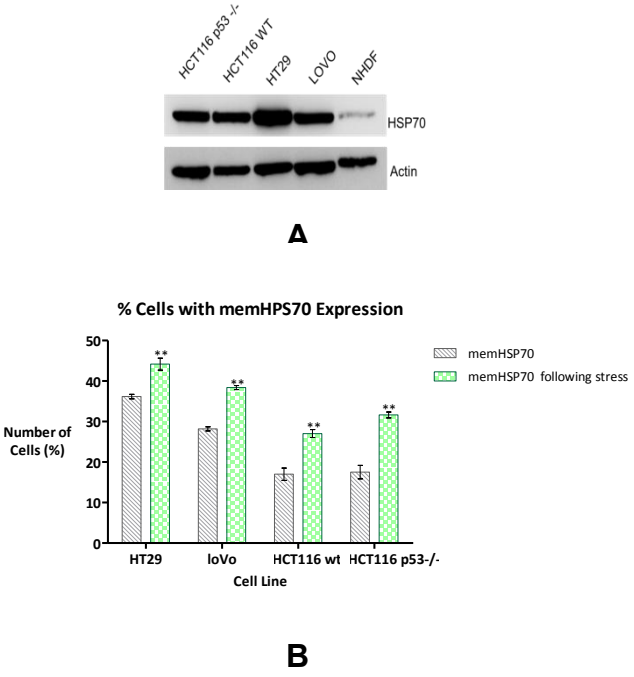

Figure 3 A. Western Blot analysis of HSP70 expression in whole cell lysates of HT29, LoVo, HCT116 and HCT116 p53 -/- colorectal cancer cell lines and NHDF cells. ß-actin was used as a loading control. **B.** The surface expression of memHsp70 in the panel of colorectal cancer cell lines was assessed using the cmHSP70.1 antibody.

Given **TPP** has been demonstrated to specifically target the membrane-bound form of HSP70, memHSP70, the selective presence of memHsp70 in the four colorectal cancer cell lines was determined by flow cytometry using a unique mouse primary monoclonal antibody, cmHSP70.1. The expression profiles, as determined by mean fluorescence per sample and percentages averaged over three unique runs (n=3) Fig. 3 B, varied between the four colorectal cancer cell lines, where HT29 exhibited the highest expression at 36% and HCT116 wt the lowest at 17%. To demonstrate that expression of memHSP70 is stress responsive the four colorectal cancer cells were incubated for two hours in serum free medium. Significantly memHSP70 expression was upregulated in all cell lines subjected to stress, Fig. 3 B.

Flow cytometric analysis of apoptosis

HT29 and HCT116 wt colorectal cancer cells, were selected for further cell death analysis using the Annexin V Propidium Iodide (PI) assay as (i) HT29 represents a relatively resistant cell line and HCT116 a relatively sensitive cell line to oxaliplatin treatment as per the *in vitro* MTS cytotoxicity data (Table 1) and (ii) HT29 exhibited the highest expression of memHSP70 and HCT116 the lowest, Fig. 3 B.

In contrast to an MTS assay, which measures inhibition of cell proliferation, analysing Annexin V/PI staining by flow cytometry quantifies the population of live cells, apoptotic cells and dead cells.

As per Fig. 4, **TPP** induces little or no cell death in either cell line following 72 hours of treatment at three varying concentrations, 5, 10 and 50 μM.

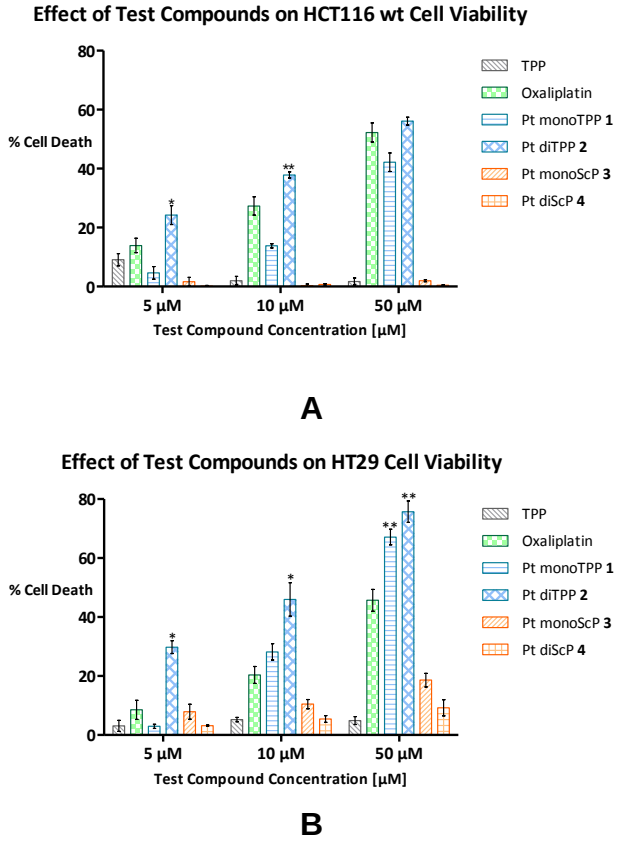

Figure 4 Flow cytometry analysis representing dead cell population (%) following 72 hour of treatment in HCT116 wt cells (**A**) and in HT29 cells (**B**) with test compounds at varying concentrations. Significant differences between the activity of Pt mono**TPP 1** and oxaliplatin, and between Pt di**TPP 2** and oxaliplatin are indicated.

Oxaliplatin, and Pt mono**TPP** and di**TPP** conjugates, **1** and **2**, clearly cause a steady increase in cell death with increasing concentration in both cell lines, Fig. 4. 50 μM oxaliplatin

treatment for example, which has an IC_{50} of 9 and 0.4 μM against HT29 and HCT116 cells respectively as determined by the MTS assay (Table 1), results in 46 % and 53 % cell death respectively post 72 hours of treatment, Fig. 4 A and B.

The Pt di**TPP** conjugate **2** causes greater cell death as compared to oxaliplatin, which causes greater cell death than the Pt mono**TPP** conjugate **1** at the three concentrations investigated against the HCT116 cells, which have the lowest expression of memHSP70 at 17 %, Fig. 4 A.

In contrast, both **1** and **2** cause significantly greater cell death as compared to oxaliplatin against the relatively resistant HT29 cell line (Fig. 4 B). It is also certainly noteworthy that the conjugates **1** and **2** exhibit far superior cytotoxicity in the HT29 cells lines, which have the highest expression of memHSP70 at 36%, as compared to the HCT116 cells at 17%. The di**TPP** conjugate **2**, is also more active as compared to the mono**TPP** conjugate **1,** as was the case against the HCT116 cells.

Pt mono**ScP** conjugate **3** and di**ScP** conjugate **4** of the scrambled peptide **ScP** (LNLETRLGFGDNKS) were developed as negative controls. Significantly both **3** and **4** caused little or no cell death in both cell lines and in particular when compared to the activity of oxaliplatin and conjugates **1** and **2** at the same concentration, Fig. 4 A and B. Therefore the scrambled peptide **ScP** when conjugated to oxaliplatin essentially negates any cytotoxicity associated with the oxaliplatin moiety which is in direct contrast to the enhanced activity observed for parent Pt **TPP** conjugates **1** and **2**.

Given **ScP** is a mostly hydrophilic and charged peptide at pH 5.7 and above, it is likely that the high molecular weight Pt conjugates of **ScP**, **3** and **4** are not readily internalised and the cell membrane acts as a barrier to their efficacy.

In turn uptake of the Pt conjugates, **1** and **2** which have the exact same hydrophilicity, charge and molecular weight as **3** and **4** respectively, must have been facilitated as previously reported for **TPP**.[3]

This data suggests that **TPP** does endow oxaliplatin, through chemical conjugation, with the potential for the targeting of cancer cells through the specific binding and internalization by memHSP70+ tumour cells via endosomal pathways. As such the breadth of this study will be expanded to more fully elucidate the *in vitro* and *in vivo* potential of **TPP** conjugation to enhance the targeting and uptake of anticancer drugs in memHsp70+ cancer cells and tumours.

Notes and references

‡ Footnotes relating to the main text should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

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