Metallodrugs for therapy and imaging: investigation of their mechanism of action

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

Publication date:
2018

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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General Discussion and Future Perspectives
In this thesis, the application of metallodrugs for therapy and imaging was investigated as part of the field of medicinal inorganic chemistry. In the introduction, an overview was given on the application of organic molecules, incorporating a metal or radiometal for either therapeutic or diagnostic purposes, particularly in relation to cancer. It is evident that their mechanism of action, their pharmacokinetic behaviour and biological targets are mostly not fully elucidated yet. Thus, our overall aims included: i) to synthesize new radiopharmaceuticals for either cancer therapy or imaging, and ii) to elucidate the mechanism of cellular uptake and excretion, the anticancer activity and the organ toxicity of some new Au containing metallodrugs in comparison to cisplatin. To investigate the toxicity and transport mechanisms of the new cytotoxic organometallic Au(III) compounds, the ex vivo model of precision cut tissue slices and human cell cultures in vitro were used.

The work described in Part A was performed at the University of British Columbia in Vancouver, BC, Canada in the Department of Medicinal Inorganic Chemistry under the supervision of Prof. Chris Orvig. Different radiotracers were developed and characterized by chemical-physical methods. Radiolabeling experiments were also performed at TRIUMF (Canada’s national laboratory for particle and nuclear physics and accelerator-based science) and in vivo animal experiments were conducted at the BC Cancer Agency.

In chapter A1, we report on the synthesis of H₄neunpa and its immunoconjugate H₄neunpa-trastuzumab (Figure 1) and showed that it can be efficiently radiolabeled with ¹¹¹In³⁺ at ambient temperature within 15 min or 30 min, respectively. ¹¹¹In is a gamma emitter and can thus be used for single photon emission computed tomography (SPECT). The immunoconjugate was further investigated in an in vivo model using HER2/neu positive subcutaneous SKOV-3 ovarian cancer xenografts bearing mice. Unfortunately, our results showed an unexpected lower accumulation of ¹¹¹In-neunpa-trastuzumab into the tumor compared to the gold-standard ¹¹¹In-CHX-DTPA-trastuzumab, which was supported by Immuno-SPECT images taken after 1 day, 3
days and 5 day post injection. Consequently, $^{111}$In-neunpa-trastuzumab does not seem to be suitable for clinical application, although *in vitro* experiments (immunoreactivity, radiolabeling efficiencies, stability in human serum) showed similar or superior properties of this chelator compared to the gold-standard. A reason for this lower accumulation might be a difference in internalization process of the chelator-HER2/neu-receptor complex. Finally, radiolabeling of H$_4$neunpa with $^{177}$Lu, a therapeutic radiometal, was tried but appeared unsuccessful. Comparison of In$^{3+}$ (92pm CN=8) and Lu$^{3+}$ (103pm CN=9)$^1$ leads to the hypothesis that either Lu$^{3+}$ might be too big for H$_4$neunpa or the preferred coordination number is not saturated because of steric hindrance.

![Figure 1. Summarized radioconjugates derived from H$_4$neunpa-p-Bn-NO$_2$.](image)

On the other hand, $^{225}$Ac and $^{213}$Bi radiolabeling of H$_4$neunpa-p-Bn-NO$_2$ was successful, as described in chapter A2. $^{225}$Ac and $^{213}$Bi are
alpha emitters that can be used for targeted alpha therapy (TAT). Additionally, this chelator could be conjugated to a PSMA targeting molecule, Glu-ureido-Lys, resulting in H₄neunpa-PSMA-L (Figure 1). Radiolabeling of H₄neunpa-PSMA-L with ¹¹¹In was disappointingly low, which could possibly be explained by the short distance between Glu-ureido-lys and the chelation cavity of H₄neunpa, thereby chelating the ¹¹¹In by the carboxylic acids of Glu-ureido-lys. Furthermore, metallacage-ligand linkage to H₄neunpa and incorporating La³⁺ was successful and fluorescence spectroscopy with La³⁺ gave promising results, showing better fluorescent properties of La-neunpa-metallacage-ligand compared to H₄neunpa-metallacage-ligand without La³⁺.

Antimony complexation reactions gave promising results as well, making the complex suitable for targeted radiotherapy, since ¹¹⁹Sb is an Auger emitter. Overall, the results in chapter A1 and chapter A2 show that H₄neunpa can be considered as an excellent bifunctional chelator. Modifications can be done easily, depending on the target molecule and radiometal of interest. It was also found that radiolabelling is dependent on the size of the biomolecule that is coupled as targeting moiety. Changing the biomolecule from the antibody trastuzumab to smaller biomolecules with a shorter linker (eg. Glu-ureido-Lys) resulted in different radiolabelling efficiencies. The work on this chelator is currently continued. As the synthesis of H₄neunpa is a 10 step reaction with a 2.3 % overall yield and difficult to apply for clinical use, a faster synthetic protocol was developed, by which H₄neunpa can be synthesized in only 4 steps (Figure 2).
In future work, the neunpa-PSMA-L could be improved by modifications of the length of the linker between Glu-ureido-Lys and H₄neunpa. The complexation with radioactive antimony would allow performing radiolabeling experiments with this radioisotope.

As $^{89}$Zr⁴⁺ is a promising radiometal for PET imaging, two hydroxamic acid bearing ligands have been synthesized as described in chapter A3, that were tested for their toxic effects towards several cancer cell lines. The synthesis of these two hydroxamic acid bearing ligands was successful and chemical-physical analysis supported this. Unfortunately, $^{89}$Zr radiolabeling was unsuccessful, possibly due to the inflexible hydroxamic acids arms as calculated with density functional theory (DFT). These findings are probably also the reason for the low toxicity of these compounds in cancer cell lines, as they are also not able to bind essential cations (eg. Cu²⁺ and Fe³⁺) in a stable manner, which is essential for the toxicity of these compounds. Decreasing
emission bands in UV-VIS experiments confirmed the instability of the Fe-complexes. In the future, DFT calculations should be performed before a set of compounds is synthesized, as done for the second generation of hydroxamic acid bearing ligands, as proposed in chapter A3. In the future, after the successful synthesis and characterization of these new hydroxamic acid bearing ligands, radiolabeling experiments with $^{89}$Zr as well as evaluation of their stability in human serum needs to be performed.

In chapter A4, we reported on a project which aim was to synthesize a bifunctional chelator H$_2$dedpa with a thiol reactive moiety for conjugation with FXa (factor Xa, a component of the blood coagulation cascade) in order to localize blood clots in patients. Our results showed that the synthesis of a bifunctional chelator H$_2$dedpa that bears a thiol reactive moiety for FXa conjugation was difficult. Three different approaches were unsuccessful, but the fourth attempt was successful and resulted in the synthesis of H$_2$dedpa-acrylate as proven by various chemical-physical techniques. Unfortunately due to time restrictions it was not possible to further investigate this molecule.

Part B was investigated at the University of Groningen under the supervision of Prof. Geny Groothuis and co-supervision of Prof. Angela Casini. The anticancer effects on various human cancer cell lines, kidney toxicity and accumulation mechanisms of several novel Au(III) cyclometallated compounds compared to cisplatin were studied.
In part B1, the state of knowledge of transport mechanisms of cisplatin and other metallodrugs was reviewed with the conclusion that there is a substantial lack of knowledge on the accumulation mechanisms of cisplatin and of the new generation anticancer metallodrugs at the molecular level. Such knowledge is necessary to elucidate the balance between activity and toxicity profiles of metal compounds. Furthermore, resistance mechanisms often involve drug transporter expressions in the targeted cells.\textsuperscript{2,3}

Many experiments to study these transport mechanisms were performed in cells \textit{in vitro} and studies performed in \textit{ex vivo} or \textit{in vivo} models are rare. Based on these studies in cell cultures, the transporter proteins OCT2 and CTR1 are hypothesized to be involved in the uptake of cisplatin into the cells, especially in kidney cells and may be responsible for kidney accumulation and severe nephrotoxic side-effects. APT7A/B and MATE are efflux transporters likely to be involved in the efflux of cisplatin and other Pt(II) drugs (Figure 3). Furthermore, other studies also support the involvement of passive diffusion mechanisms.

![Drug transporters possibly involved in cisplatin accumulation.](image)

**Figure 3.** Drug transporters possibly involved in cisplatin accumulation.
In chapter B2, a set of organometallic Au(III) compounds featuring bidentate C^N type of ligands were synthesized and characterized as well as studied for their anticancer properties in different human cancer cell lines in comparison to non-tumorigenic cells. Among the various Au compounds developed as anticancer agents, the use of cyclometallated complexes is advantageous due to redox and thermodynamic stability. Additionally, their lipophilic character can be tuned by the modification of the ancillary ligands or steric and electronic properties can be easily tuned by modification of the anionic cyclometallated ligands. Overall, our study shows the potential for improvement of the biological properties (like toxicity or PARP-1 inhibition) of organometallic gold-based compounds by tuning their coordination environment by changing the chlorido ligand to a PTA moiety to increase its water solubility or glucose moieties to target GLUT1 transporter.

![Figure 4. Au(III) organometallic complexes discussed in this thesis.](image)

The most active Au(III) compound \([\text{Au(py}^\text{b}-\text{H})(PTA)\text{Cl}]\text{PF}_6\) (PTA=1,3,5-triazaphosphaadamantane) was chosen for further characterization of the toxicity and transport mechanisms of the compound in an ex vivo model of precision cut kidney slices (PCKS) in chapter B3. We found, that this novel Au(III) compound (Figure 4)
shows also markedly toxic effects on PCKS under the selected experimental conditions after 24h incubation compared to cisplatin. Both compounds induce a concentration dependent decrease in viability of PCKS, which correlated with the Au or Pt content, respectively. The Au(III) compound showed lower TC_{50} values compared to cisplatin, being 4.3 ± 0.2 µM and 17 ± 2.0 µM respectively.

Additionally, this new experimental metallodrug was studied for its mechanism of transport and cellular accumulation in kidney slices in comparison to cisplatin. Using cimetidine as an inhibitor for OCT2 and MATE, we showed that there is no evidence that either the Au(III) compound or cisplatin are transported via OCT2 or MATE. In the case of cisplatin, this is in contrast to previously reported results. As commented in chapter B3, this may be due to the important differences between the previously reported cell-based models/assays and our tissue culturing method. Another explanation might be that cimetidine is not only an inhibitor of OCTs and MATEs, but also an H$_2$-receptor antagonist and it can also inhibit some of the drug-metabolizing cytochrome 450 (CYP). Thus, several transporters and receptors are a target of cimetidine. Consequently it might be possible, that either cimetidine does not have a full effect on the OCTs or MATEs, since other targets have a higher affinity, or other transporters or receptors which can not be inhibited by cimetidine might have an effect on cisplatin’s or Au(III) compound’s uptake and consequently toxicity. To evaluate this, further experiments are needed. Firstly, a positive control that proofs cimetidine’s ability to block OCT and MATE transport is necessary. Secondly, a more specific inhibitor of OCTs or MATEs should be studied, but to our knowledge, no specific inhibitor is available yet. Metal quantification in tissues was also achieved by ICP-MS, while histomorphology studies allowed providing evidence of the damage of specific cell types, namely distal tubular cells compared to proximal tubular cells for cisplatin. Since the Au(III) compound seems to be very toxic, we need to prevent these toxic effects by targeting it more specifically to the cancer tissue. This can be tried by linking it to
peptides or antibodies with affinity for the cancer cells as described for radiopharmaceuticals.

In chapter B4, the involvement of OCT2/MATE and CTR1/ATP7A/B in several cancer cell lines was studied for another C^N Au(III) compound, named Au(III) compound 1, containing a fluorescent coumarin moiety which makes it suitable for fluorescent microscopy. Using cimetidine as an inhibitor for OCT2/MATE and CuCl₂ as a competitor for CTR1/ATP7A/B, we studied the influence of these transporters on the Au(III) compound 1 accumulation in A2780 and A2780cisR (A2780 cells resistant for cisplatin) cells compared to cisplatin. The Au(III) compound 1 seems to be more potent than cisplatin as concluded from the IC₅₀ results after 24h and 72h incubation and also showed a higher metal content accumulation via ICP-MS. Co-incubation with CuCl₂ increased the toxicity of the Au(III) compound 1 in both A2780 and A2780cisR cells. These findings are supported by an increase in Au and Cu content, leading to the hypothesis that not only Au(III) accumulation but also Cu accumulation might be the reason for the increased toxicity or that CuCl₂ inhibits efflux pumps involved in Au(III) accumulation. A direct involvement of the CTR1 or OCT2 in the uptake of the drug could not be shown by inhibition by CuCl₂ or cimetidine. Cisplatin showed a similar behavior. After 72h, co-incubation of cisplatin with either cimetidine or CuCl₂ resulted in an increase in toxicity, but no increase in Pt content could be observed in both cell lines by ICP-MS. This result, together with the evidence for increased Cu content in A2780 cells, leads to the hypothesis that copper accumulation is the reason for the increased toxicity in these cell lines.

Overall, both research parts A and B are examples of multidisciplinary collaborations. For radiopharmaceuticals, an expertise network of radiopharmacists (incl. radiochemists) and nuclear medicine physicians is essential for meeting the needs in clinic and patient care as well as taking benefit from the fundamental knowledge of chemists and biologists. In my opinion, such collaborations are ongoing, but they could be intensified and extended.
in order to accelerate the development of new and better radiopharmaceuticals and prevent an inefficient use of money. Finally, taking into account the applications of radiotracers in the clinic, macrocyclic chelators like DOTA or NOTA are still preferred over acyclic chelators, since they show better stability in patients or superior tumor uptake.

Concerning transporter studies in vitro, ex vivo and in vivo, specific inhibitors for the tested transporter should be used in order to be able to make clear conclusions about the results of transporter inhibition experiments. It should be kept in mind that drug transporters are expressed differently in species and should be taken into account when translating from animal data to human. Moreover differences in transporter and metabolizing enzyme expression between tissues and cell types should be taken into account when evaluating accumulation and toxic effects in cancer cells and different organs.

To conclude, the topic of metallodrugs for therapy and imaging was successfully investigated in this thesis. Two different parts (A and B) focus in detail on radiopharmaceuticals for imaging/diagnosis of specific cancer types and on the mechanisms of transport as well as toxicity of new experimental Au(III) metallodrugs in vitro as well as ex vivo, respectively.

References

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