Relatório Trienal de Actividade[1]

Novembro de 2014 - Fevereiro de 2017

João Domingos Galamba Correia

Investigador Principal

Departamento de Engenharia e Ciências Nucleares

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Centro de Ciências e Tecnologias Nucleares

CAMPUS TECNOLÓGICO E NUCLEAR

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[1]No âmbito do cumprimento das obrigações decorrentes da nomeação definitiva estabelecidas no Artigo 41º do Decreto-Lei nº 124/99, de 20 de Abril.

Índice

1. Dados Pessoais

- **Nome**: João Domingos Galamba Correia
- **Naturalidade**: Porto Amélia, Moçambique
- **Nacionalidade:** Portuguesa
- **Data Nascimento**: 14 de Outubro de 1967
- **Residência**: Rua Tomás Ribeiro 45, 6º Dtº, 1050-225 Lisboa
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1.1. Graus Académicos

1993–1996 - Doutoramento em Química no Instituto de Química Inorgância da Universidade Técnica de Munique, Alemanha. Título da Tese: "*Molecular Rhenium Oxides as Oxidation Catalysts*". Aprovado com a classificação final "*Sehr Gut Bestanden*".

1985-1991 - Licenciatura em Ciências Farmacêuticas, Ramo Farmácia Industrial, na Faculdade de Farmácia da Universidade de Lisboa com estágio de pré-licenciatura nos Laboratórios Pfizer, Coina, Portugal. Média final de 16 valores.

1.2. Percurso Científico

03/2006-… - *Investigador Principal* no Departamento de Engenharia e Ciências e Nucleares (DECN) e Centro de Ciências e Tecnologias Nucleares (C^2TN) do Instituto Superior Técnico, Universidade de Lisboa.

11/2000-02/2006 - *Investigador Auxiliar Convidado* na Unidade de Ciências Químicas e Radiofarmacêuticas do ITN, Sacavém, Portugal.

01/1998-10/2000 - Bolseiro de Pós-Doutoramento (FCT-PRAXIS) na Unidade de Ciências Químicas e Radiofarmacêuticas do ITN, Sacavém, Portugal.

2. Actividade Científica

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Scopus Author ID: 7202364104

ResearcherID: J-7036-2013

Grupo: http://c2tn.tecnico.ulisboa.pt/en/research/research-groups/radiopharmaceutical-sciences

h-index (Março de 2017): 23

O trabalho científico desenvolvido no Grupo de Ciências Radiofarmacêuticas do Centro de Ciências e Tecnologias Nucleares (**C^2TN**) do Instituto Superior Técnico, tal como no triénio anterior, inseriu-se na estratégia geral do grupo, cujo objectivo principal é a concepção, síntese e caracterização de ferramentas radioactivas específicas, de natureza molecular ou "*nano*", com propriedades biológicas adequadas para aplicações de diagnóstico e/ou terapia em Medicina Nuclear. De referir o esforço despendido no domínio da imagiologia molecular, nomeadamente na concepção de sondas moleculares radioactivas capazes de detectar alvos moleculares a nível celular, visualizando-se assim alterações metabólicas que precedem as alterações morfológicas. Esta possibilidade é de crucial importância no domínio da oncologia.

Paralelamente à descoberta de novas ferramentas radioactivas, é de referir o estudo dos seus mecanismos de acção, assim como a tentativa de descoberta de alvos inovadores para imagem e/ou terapia em associação com outros grupos de investigação na área da biomedicina. Esse objectivo só será alcançado com o contributo individual de uma larga equipa multidisciplinar que abarque diferentes áreas científicas, tais como a química medicinal, radioquímica, radiofarmacologia, bioquímica, medicina e biologia, para citar apenas algumas das mais relevantes.

Os resultados obtidos no âmbito da actividade científica realizada no triénio 2014 - 2017 deram origem a 2 capítulos de livros, 10 artigos em revistas internacionais da especialidade com arbitragem e 13 Comunicações (5 orais e 8 em forma de Poster) em conferências e simpósios nacionais e internacionais.

Apresenta-se seguidamente um sumário das actividades científicas desenvolvidas em cada uma das linhas de investigação onde estive envolvido, destacando-se os resultados mais relevantes alcançados em cada uma delas.

2.1. Bisfosfonatos para imagiologia e terapia de metástases ósseas

O trabalho nesta linha temática foi realizado no âmbito de dois projectos financiados pela Fundação para a Ciência e a Tecnologia (FCT) em colaboração com o Grupo de Investigação em Oncologia Clínica Aplicada do Prof. Luís Costa do Instituto de Medicina Molecular (IMM), Faculdade de Medicina, Universidade de Lisboa:

- PTDC/QUI-QUI/115712/2009, *Synthesis, Characterization and Biological Assessment of Multi-Functional Bone-Seeking Agents*. O projecto terminou oficialmente em Fevereiro de 2014, mas alguns do resultados só foram publicados posteriormente.

- EXCL/QEQ-MED/0233/2012, *Molecular and Nano Tools for Cancer Theranostics*. O projecto terminou oficialmente em Novembro de 2016.

Na continuação do trabalho iniciado e desenvolvido no triénio anterior, concluiu-se a (radio)síntese, caracterização e avaliação biológica de uma família de complexos organometálicos do tipo *fac*-[$M(CO)_3(k^3\text{-}L)$] (M = $^{99m}Tc/^{nat}Re/^{188}Re$) com propriedades osteotrópicas. Os compostos são estabilizados por quelatos bifuncionais do tipo pirazolo-diamina contendo uma unidade bisfosfonato (pamidronato ou alendronato). Após estudos de biodistribuição em ratinhos normais, confirmou-se que os compostos radioactivos de ^{188}Re se acumulavam preferencialmente no osso. Os estudos de internalização e citotoxicidade em células tumorais mostraram que alguns complexos são internalizados, exercendo uma acção radiotóxica muito superior à do anião perrenato $[^{188}ReO_4]^-$, considerado a molécula controlo. Foi ainda possível concluir que essa acção promovia alterações morfológicas nas células e provocava danos ao nível do DNA. Estes resultados são detalhadamente descritos no seguinte artigo:

- *Novel ^{188}Re Multi-Functional Bone-Seeking Compounds: Synthesis, Biological and Radiotoxic Effects in Metastatic Breast Cancer Cells*, C. Fernandes, S. Monteiro, A.

Belchior, F. Marques, L. Gano, J. D. G. Correia, I. Santos, *Nucl. Med. Biol.* **2016**, *43*, 150-157. DOI:10.1016/j.nucmedbio.2015.11.004.

Relativamente ao desenvolvimento de novas plataformas "*nano"* decoradas com bisfosfonatos para entrega selectiva de fármacos e/ou radionuclídeos para imagem e/ou terapia de metástases ósseas, iniciaram-se estudos preliminares de preparação e caracterização de micelas simples, ainda sem a unidade bisfosfonato à superfície, com ou sem um agente citotóxico (docetaxel) no seu núcleo. Os estudos de avaliação biológica demonstraram que as micelas com docetaxel apresentavam uma acção anti-proliferativa em linhas celulares de tumores superior à do docetaxel livre para a mesma concentração de fármaco. Os estudos de biodistribuição em ratinhos saudáveis com micelas marcadas com "$^{99m}Tc(CO)_3$" demonstraram que as micelas apresentam propriedades farmacocinéticas adequadas para entrega de fármacos. Neste momento, desenvolvem-se esforços no sentido de se decorarem as micelas com bisfosfonatos para lhes conferir propriedades osteotrópicas. Os resultados aqui sumariamente descritos foram parcialmente publicados no seguinte artigo:

- *Radiolabeled Block Copolymer Micelles for Image-guided Drug Delivery*, E. Ribeiro, I. Alho, F. Marques, L. Gano, I. Correia, J. D. G. Correia, S. Casimiro, L. Costa, C. Fernandes, I. Santos, *Int. J. Pharm*. **2016**, *515* (1-2), 692-701. DOI: 10.1016/j.ijpharm.2016.11.004.

O trabalho iniciado no triénio anterior relativo à utilização das propriedades osteotrópicas dos bisfosfonatos para dirigir selectivamente complexos metálicos de platina para as metástases ósseas, efectuado no âmbito um projecto bilateral de colaboração com a Faculdade de Ciências da Universidade de Madrid, Espanha (Acciones integradas-España e E-23/12 Projecto de Acção, Portugal, PRI-AIBPT-2011-0980), resultou na elaboração de um manuscrito a ser submetido a uma revista internacional da especialidade com revisão por pares (**Anexo I**).

2.2. (Radio)péptidos para teranóstica do cancro

No âmbito de um projecto de colaboração internacional com o Department of Inorganic and Analytical Chemistry, University of Debrecen, Hungary, sintetizou-se e caracterizou-se um

complexo heterobimetálico para teranóstica do cancro do tipo $^{nat}Ga/^{67}Ga$-NODA-GA-[(η^6-Tyr-RuCp)-HAVAY-NH_2], contendo uma sequência peptídica específica para as caderinas N- e E-, sobreexpressas em determinados carcinomas. Estudos de avaliação biológica demonstraram que os complexos de ^{67}Ga são internalizados sem contudo apresentarem acção citotóxica relevante. Os resultados deste estudo foram já publicados no seguinte artigo:

- *Synthesis, Characterization and Biological Evaluation of a ^{67}Ga-Labeled (η^6-Tyr)Ru(η^5-Cp) Complex with the HAV motif*, Z. Bihari, F. Vultos, C. Fernandes, L. Gano, I. Santos, J. D. G. Correia, P. Buglyó, *J. Inorg. Biochem.* **2016**, *160*, 189-197. DOI:10.1016/j.jinorgbio.2016.02.011.

No âmbito do projecto bilateral com a Universidade de Madrid já referido anteriormente e de um projecto com a Drª Angela Casini da Universidade de Cardiff desenvolveram-se um conjunto de complexos de platina e ruténio, respectivamente, contendo a sequência peptídica ArgGlyAsp (RGD) para entrega selectiva do agente metálico citotóxico a células tumorais com sobreexpressão da integrina $\alpha_v\beta_3$. A síntese e caracterização dos compostos, bem como a sua avaliação biológica em linhas tumorais específicas foi detalhadamente descrita nos seguintes artigos:

- *Non-conventional trans-Platinum Complexes Functionalized with RDG Peptides: Chemical and Cytototoxicity Studies*, M. A. Medrano, M. Morais, V. F. Ferreira, J. D. G. Correia, A. Paulo, I. Santos, A. A. Valdes, A. Casini, F. Mendes, A. G. Quiroga, *Eur. J. Inorg. Chem.* **2017**, *in press.*

- *Functionalization of Ruthenium(II) Terpyridine Complexes with Cyclic RGD Peptides to Target Integrin receptors in Cancer Cells*, E. M. Hahn, N. Estrada, J. Han, V. F. C. Ferreira, T. G. Kapp, J. D. G. Correia, A. Casini, Fritz E. Kühn, *Eur. J. Inorg. Chem.* **2016**, *in press.*

Os resultados dos trabalhos acima mencionados foram escritos e publicados na sequência dos estágios científicos de curta duração nos laboratórios do C^2TN dos estudantes de doutoramento Z. Bihari e E. M. Hahn da University of Debrecen e University of Groningen,

respectivamente, ao abrigo de Short Term Scientific Missions (STSM´s) da Acção COST CM1105 – Functional metal complexes that bind to biomolecules.

2.3. Péptidos translocadores

Ao abrigo de um projecto de cooperação com o grupo do Prof. Miguel Castanho do IMM, Faculdade de Medicina, e da Faculdade de Medicina Veterinária, ambas da Universidade de Lisboa, cujo objectivo principal é o desenvolvimento de novos vectores peptídicos capazes de atravessar a barreira hemato-encefálica, preparámos uma família alargada de novos conjugados peptídicos que foram marcados com os radiometais ^{99m}Tc e ^{67}Ga. A avaliação biológica *in vitro* e *in vivo* dos radiopéptidos revelou que um dos péptidos (PepH3) apresentava características importantes para funcionar como "shutle" para transporte bidireccional de "carga" para o cérebro. Os resultados já obtidos foram compilados num artigo que foi recentemente aceite numa revista da especialidade de alto impacto:

- *Novel peptides derived from Dengue virus capsid protein translocate reversibly the blood-brain barrier through a receptor-free mechanism*, V. Neves, F. Aires-da-Silva, M. Morais, L. Gano, E. Ribeiro, A. Pinto, S. Aguiar, D. Gaspar, C. Fernandes, J. D. G. Correia, M. Castanho, *ACS, Chemical Biology* **2017**, *in press.*

2.4. Fragmentos de colagénio

Os fragmentos de colagénio tipo I, onde se incluem moléculas mais complexas de peso molecular elevado tal como o NTX, CTX e ICTP, ou moléculas mais simples como derivados de amino ácidos (e.g. hydroxiprolina e hidroxilisina) ou derivados dos "cross-links" tais como a deoxipiridinolina (DPD) ou piridinolina (PD), são biomarcadores de remodelação óssea na monitorização da doença metastática óssea e na resposta à terapêutica de anti-reabsorção com bisfosfonatos. O trabalho que tem vindo a ser desenvolvido em cooperação com o Grupo de Investigação em Oncologia Clínica Aplicada do Prof. Luís Costa do IMM contempla essencialmente duas vertentes:

- *Avaliação do efeito biológico de moléculas sintéticas de baixo peso molecular derivadas do colagénio tipo I*: o objectivo é o estudo do efeito biológico dos isómeros da hidroxiprolina (Hyp, HO-L-proline, and HO-D-proline), hidroxilisina (Hyl) e deoxipiridinolina (DPD) nas

características funcionais (e.g. proliferação, migração e invasão) de células tumorais da mama (MDA-MB-231) e da próstata (PC-3). Desta forma será possível identificar o papel desses fragmentos nas fases iniciais da cascata metastática. Os resultados até agora obtidos mostraram que os isómeros da hidroxiprolina e a a Hyl não induziram efeito significativo na proliferação celular. A DPD apresentou um efeito anti-proliferativo moderado na linha celular PC-3. O mesmo composto apresentou efeito contrário na linha MDA-MB-231. Os compostos Hyl e DPD não influenciaram a migração e invasão de ambas as linhas. Em conclusão, a DPD é o único fragmento em que se detecta algum efeito biológico, nomeadamente na proliferação celular, o que potencialmente pode afectar a carga tumoral nas doenças avançadas de mama e de próstata. Este trabalho serviu de base à elaboração de uma Tese de Mestrado apresentar brevemente: Bárbara Franco Andrade Góis - Título da tese: "*The effect of bone collagen fragments on breast and prostate cancer cells*", Mestrado em Engenharia Biomédica, Instituto Superior Técnico e Faculdade de Medicina, Universidade de Lisboa, 2017.

- *Avaliação do efeito biológico de moléculas de peso molecular elevado derivadas do colagénio tipo I*: tal como na vertente anterior, o objectivo do trabalho é estudar o efeito biológico de fragmentos de elevado peso molecular (e.g. NTX, CTX e ICTP) derivados do colagénio de Tipo I nas características funcionais de células tumorais. Tendo em consideração a sua natureza diversa e dificuldade de síntese optou-se por isolar os fragmentos mencionados a partir de osso humano por digestão com proteases específicas. Após purificação dos extractos por cromatografia em gel, foram identificadas as fracções contendo os fragmentos de interesse. Neste momento procede-se à identificação/caracterização dos fragmentos isolados por espectrometria de Massa em colaboração com a Drª Ana Coelho do ITQB, Universidade Nove de Lisboa. O trabalho aqui sumariamente descrito serviu de base à submissão conjunta de uma bolsa de pós-doutoramento (Drª Irina Alho).

2.5. Detecção *in vivo* do Óxido Nítrico Sintase (NOS)

Este tópico tem vindo a ser desenvolvido com base no projecto FCT: *Nitric Oxide Synthase targeting with Re(I)/^{99m}Tc(I)-complexes containing L-Arg derivatives: A structure-activity study* - PTDC/QUI-QUI/121752/2010 (2012 - 2015). Ao abrigo deste projecto foi realizado

trabalho que resultou na elaboração de dois artigos em revistas internacionais, um dos quais já publicado e outro em vias de submissão:

- *Re(I) and Tc(I) Complexes for Targeting Nitric Oxide Synthase: Influence of the Chelator in the Affinity for the Enzyme*, B. L. Oliveira, M. Morais, F. Mendes, I. S. Moreira, C. Cordeiro, P. A. Fernandes, M. J. Ramos, R. Alberto, I. Santos, J. D. G. Correia, *Chem. Biol. Drug Des.* **2015**, *86*, 1072-1086. DOI:10.1111/cbdd.12575.

Neste trabalho sintetizaram-se e caracterizaram-se complexos organometálicos de Re(I)/^{99m}Tc(I) estabilizados pela unidade quelante diamino-propionato contendo derivados da L-arginina capazes de interagir com a Óxido Nítrico Sintase induzida (iNOS). Os estudos enzimáticos realizados revelaram que os complexos obtidos apresentam um menor capacidade de interacção com o enzima quando comparados com os complexos estabilizados pela unidade pirazolo-diamina estudados anteriormente. Esta diferença, pode ser parcialmente explicada com base nos parâmetros estruturais envolvidos na interacção dos complexos com o local activo do enzima. Assim, tendo como objectivo clarificar as interacções específicas na ligação proteína (enzima)/ligando (complexos organometálicos), quer no local de ligação do grupo guanidínio quer no canal de acesso do substrato ao enzima, realizaram-se estudos de docking e dinâmica molecular capazes de estabelecer uma relação estrutura-actividade.

- Technetium-99m complexes of L-arginine derivatives for cancer imaging, M. Morais, B. L. Oliveira, V. F. C. Ferreira, F. Mendes, P. Raposinho, I. Santos, J. D. G. Correia, *Dalton Trans.*, submitted (**Anexo II**).

Neste trabalho desenvolveram-se complexos organometálicos de Re(I)/^{99m}Tc(I) contendo derivados da L-arginina que são capazes de atravessar a membrana celular e de se acumularem no citoplasma. Estudos mecanísticos preliminares sugerem que a entrada na célula é mediada por transportadores de aminoácidos, nomeadamente o transportador system y^+.

2.6. Fragmentos de anticorpos

Ainda na sequência do projecto de colaboração com o Grupo do Prof. João Gonçalves da Faculdade de Farmácia da Universidade de Lisboa (*Albumin binding-domain fusions to improve protein pharmacokinetics: PTDC/SAU-FAR/115846/2009*) foi aceite recentemente para publicação o seguinte artigo:

- *Albumin-binding domain from Streptococcus zooepidemicus protein Zag as a novel strategy to improve the half-life of therapeutic proteins*, C. Cantante, S. Lourenço, M. Morais, J. Leandro, L. Gano, N. Silva, P. Leandro, M. Serrano, A. O. Henriques, C. Fontes, J. D. G. Correia, F. Aires-da-Silvab, J. Gonçalves, *Journal of Biotechnology* **2017**, *in press*.

Os resultados dos estudos de biodistribuição em ratinhos saudáveis com as proteínas marcadas com ^{99m}Tc permitiram concluir inequivocamente que a fusão de domínios de ligação à albumina de origem bacteriana, neste caso o ZAG, a fragmentos de anticorpos conduz a um aumento da semi-vida plasmática da proteína resultante. Desta forma é possível melhorar as propriedades farmacocinéticas deste tipo de biofármacos, conduzindo a um aumento do seu potencial terapêutico sem afectar as propriedades específicas de ligação do anticorpo, tal como já se tinha concluído anteriormente após marcação de fragmentos de anticorpos com ^{67}Ga seguido de avaliação biológica.

2.7. Partículas do tipo viral (VLP) para entrega selectiva de radionuclídeos

O objectivo geral do projecto é avaliar a possibilidade de utilizar nano-plataformas multimodais baseadas em partículas do tipo viral (VLPs) como transportadores de fármacos e/ou radionuclídeos citotóxicos para aplicações na teranóstica do cancro. O vírus da imunodeficiência humana (HIV) manipulado foi seleccionado como protótipo de VLP e o Receptor do Factor de Crescimento Epidérmico Humano 2 (HER2) como alvo. A cápsula proteica do HIV será modificada de forma a expressar à sua superfície fragmentos de anticorpos com elevada afinidade e especificidade para o HER2. Assim, será feita uma abordagem estrutural multidisciplinar, combinando métodos computacionais e experimentais para investigação desses sistemas à nano-escala. A investigadora pós-doutorada Rita Melo, em estreita colaboração com a Drª Irina Moreira do Centro de Neurociências e Biologia

Celular da Universidade de Coimbra, iniciou um estudo de modelação computacional e simulações por dinâmica molecular (MD) da interacção de anticorpos específicos anti-HER2 e o Receptor do Factor de Crescimento Epidérmico Humano 2 (HER2).

Deste projecto resultou já a publicação de um artigo em revista internacional:

- *A Machine learning approach for hot-spot detection at protein-protein Interfaces*, R. Melo, R. Fieldhouse, A. Melo, J. D. G. Correia, M. N. D. S. Cordeiro, Z. H. Gümü¸ J. Costa, A. M. J. J. Bonvin, I. S. Moreira, *International Journal of Molecular Sciences* **2017**, *17*(8), 1215. doi:10.3390/ijms17081215

3. Projectos de investigação

Investigador Responsável

- **Projecto HOVIONE/IST** (**16/03/2016-…**): *Synthetic Process Development and Analytical Characterization of Peptide Sequences*.

Membro da equipa:

- *Desenvolvimento de péptidos translocadores da barreira hematoencefálica novas moléculas terapêuticas para sistema nervoso central* - PTDC/BBBNAN/1578/2014 (2016 – 2019) - Investigador responsável: Doutora Vera Luisa Santos Neves (IMM/Faculdade de Medicina de Lisboa).

- *Ultrapassando o dilema da entrega de fármacos no cérebro: Desenvolvimento de anticorpos de domínio único para direccionamento e entrega de drogas no cérebro* - PTDC/BBBBIO/0508/2014 (2016 – 2019) - Investigador responsável: Doutor Frederico Nuno Castanheira Aires da Silva (Faculdade de Medicina Veterinária, Universidade de Lisboa).

- *Sistemas Moleculares e Nano para Teranóstica de Cancro* - EXCL/QEQ-MED/0233/2012 (2013 – 2016) - Investigador responsável: Doutora Isabel Rego dos Santos (C^2TN).

- *Alvejamento Duplo de Tumores EGFR positivos* - EXPL/QEQ-MED/1950/2013 (2014 – 2015 – Investigador Responsável: Doutora Célia Fernandes (C^2TN).

- *Radiolabeling and biological assessment of therapeutic antibodies* – Technophage-IMM. Services Agreement, celebrado entre o IST/ITN e a empresa TECHNOPHAGE.

- Participação, como representante do C^2TN/IST, na elaboração da proposta de candidatura da PPBI - Plataforma Portuguesa de BioImagem. Aprovada com sucesso em 2014 (1ª fase) e financiamento aprovado em Fevereiro de 2017 (2017).

- **COST Action CM1004** - *Synthetic Probes for Chemical Proteomics and Elucidation of Biosynthetic Pathways*. Representante nacional.

- **COST Action CM1105** – *Functional Metal Complexes that Bind to Biomolecules*.

- **COST Action TD1004** - *Theranostics Imaging and Therapy: An Action to Develop Novel Nanosized Systems for Imaging-Guided Drug Delivery*.

4. Supervisão de trabalhos de investigação

4.1. Teses de Licenciatura/Mestrado

Licenciatura

- **Mariana Antunes**, *Síntese de polipéptidos baseados em glutamina e estudo da sua associação em fase aquosa*, Química, Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa, 2016.

Mestrado (em curso)

- **Rúben Diogo Marques da Silva** - Título da tese: "*Study of the impact of metalophilic hydrogelators on polyglutamine aggregation*", Mestrado em Bioquímica, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2017.

- **Bárbara Franco Andrade Góis** - Título da tese: "*The effect of bone collagen fragments on breast and prostate cancer cells*", Mestrado em Engenharia Biomédica, Instituto Superior Técnico e Faculdade de Medicina, Universidade de Lisboa, 2017.

4.2. Visitantes estrangeiros

Estudantes de doutoramento

- **Eva M. Hahn**, Zentralinstitut für Katalyseforschung, Technische Universität München, Munich, Germany. *Short Term Scientific Mission* no âmbito da Acção COST CM 1105, Functional Metal Complexes that Bind to Biomolecules: *Novel RGD Derivatives for Metal Complexation*. 10 de Fevereiro a 30 de Abril de 2015.

- **Zsolt Bihari**, Faculty of Science and Technology, University of Debrecen, Debrecen, Hungary. *Short Term Scientific Mission* no âmbito da Acção COST CM 1105, Functional Metal Complexes that Bind to Biomolecules: *Synthesis, Characterization and Biological Evaluation or Radiometallated (η5-Cp)Ru(η6-Tyr) Peptides with HAV motif.* 14 de Fevereiro a 14 de Março de 2015.

- **Liam Connah**, MR Neuroimaging Agents research group, Max Planck Institute for Biological Cybernetics, Tuebingen, Germany. *Short Term Scientific Mission* no âmbito da Acção COST TD1004, Theragnostics Imaging and Therapy, An Action to Develop Novel Nanosized Systems for Imaging-Guided Drug Delivery: *Synthesis of bismacrocyclic smart contrast agents (SCAs) using solid phase techniques*. 19 de Agosto a 16 de Setembro de 2015.

Investigadores (Sabbatical leave)

- **Dr. Christian Kowol**, University of Vienna, Institute of Inorganic Chemistry, Waehringer, Vienna, Austria. *Short Term Scientific Mission* no âmbito da Acção COST CM 1105, Functional Metal Complexes that Bind to Biomolecules: *Novel peptide-targeted platinum(IV) complexes*. 1 de Fevereiro a 30 de Abril de 2016.

4.3. Investigadores pós-doutorados

- Supervisor, em parceria com o Dr. Luís Costa do IMM, do trabalho de investigação da investigadora pós-doutorada Dr. Irina Duarte Alho: *Biological role of clinically relevant collagen type I fragments in bone metastatic disease* (desde 2015).

- Supervisor, em parceria com a Drª. Sandra Cabo-Verde e a Drª Irina Moreira respectivamente do C^2TN e Univ. do Porto, do trabalho de investigação da bolseira de Pós-doutoramento Rita Paiva Melo (FRH/BPD/97650/2013). Título do plano de trabalho: *Target-specific delivery of radioactivity to cancer cells by virus-like particles: a computational chemistry and bioengineering approach*.

- Supervisor, em parceria com a Prof. Maria João Romão da FCT-UNL, do trabalho de investigação da bolseira de Pós-doutoramento Márcia Alexandra da Silva Correia (SFRH/BPD/64917/2009). Título do plano de trabalho: *Structural and Functional Studies on Nitric Oxide Synthase Complexed to* ^{99m}*Tc/Re Compounds.*

5. Actividade como docente

2010-2016 - Colaborou como Professor convidado na docência da Unidade Curricular "*Química Radiofarmacêutica*" do **Curso de Mestrado em Química Farmacêutica e Terapêutica da Faculdade de Farmácia**, Universidade de Lisboa. Títulos das aulas: "*Moléculas Orgânicas Radioiodadas*" (2 h), "*Radiofármacos para Tomografia por Emissão de Positrão (PET)*" (2 h), "*Formulação e Controlo de Qualidade de Radiofármacos*" (2 h) e "*Radiofármacos Específicos – Estado Actual e Tendências Futuras*" (2 h).

2013-… - Docente convidado da Unidade Curricular "*Drug Discovery and Development in Oncology*" do **Curso de Mestrado em Oncobiologia** da Faculdade de Medicina, Universidade de Lisboa. Título Aula: "*Radiopharmaceutical Science and Cancer Therapy*" (2 h).

2014-… Docente convidado do "*Preceptorship Program in Bone metastases and bone-targeting agents*" organizado pelo Professor Luís Costa da Faculdade de Medicina da

Universidade de Lisboa, IMM e Divisão de Oncologia e Radiologia do HSM. Título Aula: "*Research with Radionuclides*"(2 h).

2015-… Docente convidado do **Curso de Mestrado em Bionanotecnologia** da Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa. Título Aula: *"(Radio)nanoparticles: Applications*" (2 h).

6. Participação em júris académicos nacionais e internacionais (Doutoramento)

Nacionais

5 - Título da tese: "*Engineered MRI nanoprobes based on superparamagnetic iron oxide nanoparticles*", Susana Isabel Conde Jesus Palma. Doutoramento em Bioengenharia (MIT-Portugal). Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa (Data da prova: 2 de Dezembro de 2015). *Orientador(a)*: Prof. Dr. Ana Cecília Roque.

4 - Título da tese: "*Peptide self-assembled materials for gas transport*", Joana Raquel de Oliveira Durão. Doutoramento em Engenharia Biomédica. Faculdade de Engenharia, Universidade do Porto (Data da prova: 8 de Julho de 2016). *Orientador(a)*: Prof. Dr. Luís Miguel Gales Pereira Pinto.

3 - Título da tese: "*Structural and functional studies on the reactivity of CORMs with plasma proteins*", Marino Filipe Alves dos Santos. Doutoramento em Bioquímica. Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa (Data da prova: 11 de Julho de 2016). *Orientador(a)*: Doutora Teresa Sacadura Santos-Silva.

Internacionais

2 - Título da tese: "*Supramolecular metallocages as potential delivery systems for anticancer drugs*", Andrea Schmidt. Doutoramento em Ciências da Natureza (Doktors der Naturwissenschaften, Dr. rer. nat.). Technische Universität München, Fakultät für Chemie, Fachgebiet Molekulare Katalyse (Data da prova: 4 de Outubro de 2016). *Orientador(a)*: Prof. Dr. Fritz. E. Kühn.

1 - Título da tese: "*Ruthenium and Gold Complexes as potential Anticancer Drugs targeting selectively Integrin Receptors*", Eva M. Hahn. Doutoramento em Ciências da Natureza (Doktors der Naturwissenschaften, Dr. rer. nat.). Technische Universität München, Fakultät für Chemie, Fachgebiet Molekulare Katalyse (Data da prova: 4 de Outubro de 2016). *Orientador(a)*: Prof. Dr. Fritz. E. Kühn.

7. Publicações

7.1. Livros ou capítulos de livros

2 - M. Morais, J. D. G. Correia, I. Santos, M. Pelecanou, I. Pirmettis, M. Papadopoulos, (2015). A new class of ^{99m}Tc(I) agents for SLND: Chemical design and synthesis. In: *Radiopharmaceuticals For Sentinel Lymph Node Detection*: Status and Trends, IAEA Radioisotopes and Radiopharmaceuticals series, no. 6, STI/PUB/1674, lSSN: 2077---6462, ISBN: 978-92---0-109714-9. Chapter 5, pp. 95-107.

1 - M. Morais, J. D. G. Correia, I. Santos, M. Pelecanou, I. Pirmettis, M. Papadopoulos, (2015). A new class of ^{99m}Tc(I) agentes for SLND: Labelling and quality control. In: *Radiopharmaceuticals For Sentinel Lymph Node Detection*: Status and Trends, IAEA Radioisotopes and Radiopharmaceuticals series, no. 6, STI/PUB/1674, lSSN: 2077---6462, ISBN: 978-92---0-109714-9. Chapter 6, pp. 109-114.

7.2. Revistas científicas internacionais com arbitragem

10 - *Novel peptides derived from Dengue virus capsid protein translocate reversibly the blood-brain barrier through a receptor-free mechanism*, V. Neves, F. Aires-da-Silva, M. Morais, L. Gano, A. Pinto, S. Aguiar, D. Gaspar, C. Fernandes, João D. G. Correia, M. Castanho, *ACS Chem. Biol.* **2016**, *in press.*

9 - *Albumin-binding domain from Streptococcus zooepidemicus protein Zag as a novel strategy to improve the half-life of therapeutic proteins*, C. Cantante, S. Lourenço, M. Morais, J. Leandro, L. Gano, N. Silva, P. Leandro, M. Serrano, A. O. Henriques, C. Fontes, J. D. G. Correia, F. Aires da Silva, J. Gonçalves, *J. Biotechnol.* **2017**, *in press*.

8 - *Non-conventional trans-Platinum Complexes Functionalized with RDG Peptides: Chemical and Cytototoxicity Studies,* M. A. Medrano, M. Morais, V. F. Ferreira, J. D. G. Correia, A. Paulo, I. Santos, A. A. Valdes, A. Casini, F. Mendes, A. G. Quiroga, *Eur. J. Inorg. Chem.* **2017**, *in press.* DOI: 10.1002/ejic.201700072.

7 - *Functionalization of Ruthenium(II) Terpyridine Complexes with Cyclic RGD Peptides to Target Integrin receptors in Cancer Cells,* E. M. Hahn, N. Estrada, J. Han, V. F. C. Ferreira, T. G. Kapp, J. D. G. Correia, A. Casini, Fritz E. Kühn, *Eur. J. Inorg. Chem.* **2016**, *in press.* DOI: 10.1002/ejic.201601094.

6 - *Radiolabeled Block Copolymer Micelles for Image-guided Drug Delivery,* E. Ribeiro, I. Alho, F. Marques, L. Gano, I. Correia, J. D. G. Correia, S. Casimiro, L. Costa, C. Fernandes, I. Santos, *Int. J. Pharm.* **2016**, *515* (1-2), 692-701. DOI: 10.1016/j.ijpharm.2016.11.004.

5 - *A Machine Learning Approach for Hot-Spot Detection at Protein-Protein Interfaces,* R. Melo, R. Fieldhouse, A. Melo, J. D G Correia, M. N. D. S. Cordeiro, Z. H. Gumus, J. Costa, A. M. J. J. Bonvin, I. S. Moreira, *Int. J. Mol. Sci.* **2016**, *17*. DOI:10.3390/ijms17081215.

4 - *Biological Assessment of Radiodinated Kyotorphin Derivatives,* M. C. Oliveira, L. Gano, I. Santos, J. D. G. Correia, M. A. Castanho, I. D. Serrano, S. S. Santos, M. Ribeiro, J. Perazzo, I. Tavares, M. Heras, E. Bardaji, *MedChemComm* **2016**, *7*, 906-913. DOI: 10.1039/C6MD00028B.

3 - *Synthesis, Characterization and Biological Evaluation of a ^{67}Ga-Labeled (η^6-Tyr)Ru(η^5-Cp) Complex with the HAV motif,* Z. Bihari, F. Vultos, C. Fernandes, L. Gano, I. Santos, J. D. G. Correia, P. Buglyó, *J. Inorg. Biochem.* **2016**, *160*, 189-197. DOI:10.1016/j.jinorgbio.2016.02.011.

2 - *Novel ^{188}Re Multi-Functional Bone-Seeking Compounds: Synthesis, Biological and Radiotoxic Effects in Metastatic Breast Cancer Cells,* C. Fernandes, S. Monteiro, A. Belchior, F. Marques, L. Gano, J. D. G. Correia, I. Santos, *Nucl. Med. Biol.* **2016**, *43*, 150-157. DOI:10.1016/j.nucmedbio.2015.11.004.

1 - *Re(I) and Tc(I) Complexes for Targeting Nitric Oxide Synthase: Influence of the Chelator in the Affinity for the Enzyme*, B. L. Oliveira, M. Morais, F. Mendes, I. S. Moreira, C. Cordeiro, P. A. Fernandes, M. J. Ramos, R. Alberto, I. Santos, J. D. G. Correia, *Chem. Biol. Drug Des.* **2015**, *86*, 1072-1086. DOI:10.1111/cbdd.12575.

7.3. Conferências

7.3.1. Comunicações Orais

5 - *The Importance of Radionuclides in Drug Development*, J. D. G. Correia, Invited Lecture at the Inorganic Chemistry Institute, Technical University of Munich, Garching b. München. Munique, Alemanha. 4 de Outubro, 2016,

4 - *Radioactive Bone-seeking Molecular and Nanoparticle Platforms for Theranostic Applications*, J. D. G. Correia, Conference on radiopharmaceutical agents to treat bone metastases, Instituto de Medicina Molecular, Lisboa, Portugal. 4 de Dezembro de 2015.

3 - New Bimodal Nanoprobes for Sentinel Lymph Node Imaging, J. D. G. Correia, XV Congresso Nacional de Medicina Nuclear, Coimbra, Portugal. 19 a 21 de Novembro de 2015.

2 - *Radiometallated L-arginine derivatives for tumor imaging*, J. D. G. Correia, 6th ECCLS - 6th European Conference on Chemistry for Life Sciences, Lisboa, Portugal. 10 a 12 de Junho, 2015.

1 - *Radiolabeled Peptide-modified Gold Nanoparticles for Cancer Theranostics*, European Molecular Imaging Meeting - EMIM 2015, Tübingen, Germany. 18 a 20 de Março, 2015.

7.3.2. Poster

8 - *Tyrosine-kinase receptor targeted platinum(IV) complexes*, C. Kowol, J. D. G Correia, P. Heffeter, W. Berger, I. Santos, B. Keppler, *4th Whole Action Meeting of the COST Action*

CM1105, 3rd International Symposium on Functional Metal Complexes that Bind to Biomolecules, Palma de Mallorca, Spain, April 28-29, 2016

7 - *In-111 labeled peptides towards the estrogen receptor for theranostic of breast cancer*, F. Vultos, M. Scheepstra, C. Fernandes, F. Mendes, L. Brunsveld, J. D. G. Correia, L. Gano, *15th Iberian Peptide Meeting – EPI XV*, 10-12 February, 2016, Porto, Portugal.

6 - *Novel radiopeptides for molecular imaging of EGFR positive tumors*, A. Gonçalves, L. Gano, J. D. G. Correia, F. Mendes, M. Morais, I. Santos, C. Fernandes, *15th Iberian Peptide Meeting – EPI XV*, 10-12 February, 2016, Porto, Portugal.

5 - *Block copolymer micelles for cancer therapy*, E. M. Ribeiro, C. Fernandes, F. Marques, J. D. G. Correia, D. Matos, I. Alho, S. Casimiro, L. Costa, I. Santos, *Training Network project Trace'N Treat, Conference on Molecular and Supramolecular Carriers for Imaging and Therapy*, 13-15th of July, 2015, Lisbon, Portugal.

4 - *In-111 labeled peptides targeting the estrogen receptor for theranostic of cancer*, F. Vultos, C. Fernandes, J. D. G. Correia, I. Santos, L. Gano, *Training Network project Trace'N Treat, Conference on Molecular and Supramolecular Carriers for Imaging and Therapy*, 13-15th of July, 2015, Lisbon, Portugal.

3 - *Synthesis, characterization and biological evaluation of radiometallated Ru(η^5-Cp)(η^6-Tyr) peptides with the HAV motif*, Z. Bihari, F. Vultos, J. D. G. Correia, C. Fernandes, L. Gano, I. Santos, P. Buglyó, *13th International Symposium on Applied Bioinorganic Chemistry (ISABC13)*, 12-15 June 2015, NUI Galway, Galway, Ireland

2 - *Influence of the radionuclide on the stability and biological profile of a ER targeting peptide*, F. Vultos, M. Belo, C. Fernandes, J. D. G. Correia, M. C. Oliveira, I. Santos, L. Gano, *Workshop LOWDOSE-PT-2015, Biological effects and risks of low dose and protracted exposures to ionizing radiation*, 15-16 April, 2015, CTN/IST, Bobadela LRS, Portugal.

1 - *Novel $^{67/68}$Ga-complexes for molecular imaging of EGFR positive tumors*, A. Gonçalves, M. Morais, L. Gano, J. D. G. Correia, F. Mendes, I. Santos, C. Fernandes, *10th Anual*

Meeting of the European Society for Molecular Imaging, European Molecular Imaging Meeting – EMIM 2015, 18-20 March, 2015, Tübingen, Germany.

8. Colaborações científicas

- Prof. Angela Casini, School of Chemistry, University of Cardiff, UK.

- Dr. Olga Iranzo, Institut des Sciences Moléculaires de Marseille, UMR 7313, Aix Marseille Université CNRS, Marseille e Instituto de Tecnologia Química e Biológica, UNL, Oeiras, Portugal.

- Prof. Adoración G. Quiroga, Departamento de Química Inorgánica, Facultad de Ciencias, Universidad Autónoma de Madrid, Espanha.

- Dr. M. Angeles Jiménez, Departamento de Química Física Biológica, Instituto de Química Física Rocasolano, CSIC, Madrid, Espanha.

- Prof. Roger Alberto, Department of Chemistry, University of Zurich, Switzerland.

- Prof. Miguel Castanho, IMM, Faculdade de Medicina, Universidade de Lisboa, Lisboa, Portugal.

- Prof. Luís Costa, IMM, Faculdade de Medicina, Universidade de Lisboa, Lisboa, Portugal.

- Dr. Irina Moreira, Faculdade de Ciências, Universidade do Porto, Porto, Portugal.

- Prof. Maria João Romão, FCT, Universidade Nova de Lisboa, Monte da Caparica, Portugal.

- Prof. Paula Gomes, Faculdade de Ciências, Universidade do Porto, Porto, Portugal.

- Prof. João Gonçalves, Faculdade de Farmácia, Universidade de Lisboa, Lisboa, Portugal.

- Dr. Frederico Aires da Silva, Technophage & Faculdade de Medicina Veterinária, Universidade de Lisboa, Lisboa, Portugal.

9. Actividade como especialista

- Membro efectivo da Comissão de Avaliação de Medicamentos do INFARMED. Desde 5 de Julho de 2010 até à presente data.

- Representante da Sociedade Portuguesa de Química na IUPAC – Divisão II (Química Inorgânica). Desde Janeiro de 2013 até à presente data.

10. Conferências, cursos e missões científicas

Cursos frequentados

- *Workshop on Immuno-imaging and molecular therapy*, Vrije Universiteit Brussel, Faculty of Medicine & Pharmacy, Bruxelas, Bélgica. 25 a 29 de Abril de 2016

Conferências/Congressos:

- *European Molecular Imaging Meeting - EMIM 2015*, Tübingen, Alemanha. 18 a 20 de Março de **2015**.

- *6th European Conference Chemistry in the Life Sciences*, Lisboa, Portugal, 10 a 12 de Junho de **2015**.

- *XV Congresso Nacional de Medicina Nuclear*, Centro Hospitalar e Universitário de Coimbra, Coimbra, Portugal. 19 a 21 de Novembro de **201**5.

- *Workshop on Imaging and Radiation Biomarkers – Thematic Strand" Radiopharmaceutical Sciences and Health Physics"* (Organizador), Centro de Ciências e Tecnologias Nucleares, Instituto Superior Técnico, Universidade de Lisboa. 13 de Novembro de **2015**.

- *34rd European Peptide Symposium (34 EPS)*, Leipzig, Alemanha. 4 a 9 de Setembro de **2016**.

- *Workshop on Nuclear Molecular Imaging*, Centro de Ciências e Tecnologias Nucleares, Instituto Superior Técnico, Universidade de Lisboa. 5 e 6 de Dezembro de **2016**.

João Domingos Galamba Correia

Bobadela LRS, 9 de Março de 2017

Anexo I

Novel structures of platinum complexes bearing N-bisphosphonates and study of their biological properties

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Dedicated to Prof. Carmen Navarro-Ranninger on the occasion of her last year at Universidad Autónoma de Madrid and in recognition of her leadership and lasting scientific contributions

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KEYWORDS: Pt complexes, Bisphosphonates, cancer

ABBREVIATIONS: Ipa: isopropylamine; BP: Bisphosphonates; PAM: Pamidronate; and ALEN: Alendronate.

Abstract

Novel bisphosphonate (BP) platinum complexes: [Pt(isopropylamine)$_2$(BP)]NO_3 (BP=Pamidronate and Alendronate) have been synthesized and characterized. Their monomeric structure contains a BP acting as chelate ligand through its oxygen atom donors, confering the compound´s cationic structure with a good solubility in water. Preliminary aquation studies showed high stability. The toxicity versus cancer cell lines and reactivity versus biological targets such as DNA (both CT-DNA and plasmid DNA) have been evaluated.

Bisphosphonates (BP's) are an effective drug class indicated for the treatment of pathologic conditions characterized by increased osteoclast-mediated bone resorption, namely Paget's disease, osteoporosis and tumor bone disease(2-4). BP´s bind strongly to hydroxyapatite, namely to the biological apatite, the main component of the inorganic matrix of bone. Such high affinity is explained by the chelation to Ca^{2+} ions. The BP's properties are assigned to cellular effects on osteoclasts, and the nature of the substituents on the basic structure of the BP is determinant for inhibition of bone resorption(5).

Recent studies suggest that BP's may have also antitumor activity, however, such finding must still be confirmed in clinical setting(6). More importantly, BP´s are also being studied to deliver anticancer drugs selectively to bone metastases(1). In this way, a reduction of the severe side effects associated to most systemic chemotherapeutic agents used in the clinical setting is expected.

Cisplatin is still one of the most potent agents currently used in cancer chemotherapy. However, patients experience side effects which have guided the investigations towards non-conventional platinum complexes. The idea of using a (bis)phosphonate within these structures will direct the cytotoxic drug specifically to the bone and/or help in the reduction of the severe side effects. Studies on this topic have shown that the antiproliferative effect of phosphonate-containing platinum(II) complexes(7) was not better than cisplatin's but they improved the BPs activity of the precursor ligand. Later work on these complexes confirmed a superior therapeutic activity in transplanted rat osteosarcoma models(8,9,10). All these examples are monomeric structures, where the BPs coordinate via nitrogen and oxygen, and the highly charged oxygen of the BPs are free to interact with their potential targets.

The interest of platinum BPs derivatives has also motivatedthe development of biomimetic apatite nanocrytals for potential use in bone implantation, which act as a local targeted delivery system for anticancer and anti-metastatic drugs(12), without however surpassing the cytotoxicity of cisplatin (13). With the aim of developing novel osteotropic platinum(II) complexes potentially useful for treating bone metastatic disease and/or to avoid the severe side effects linked to systemic treatments, we have synthesized and fully characterized two platinum(II) complexes of *cis* configuration bearing an isopropyl amine and the nitrogen-containinig BP´s pamidronate(PAM) and alendronate(ALEN) as chelating ligands . Their antiproliferative properties in different cancer cell lines and their reactivity versus biological targets such as models of DNA were also studied.

The complexes were prepared using a metathesis reaction of *cis*-Pt(isopropylamine)$_2$X$_2$ (X = Cl^-, I^-) with $AgNO_3$, allowing the reaction with PAM and ALEN to afford the monomeric complexes, **1** and **2**, respectively. The reaction was studied at different conditions, varying the stoichiometric ratio

Pt:BPs and the temperature. However, in all cases, the same monomeric compound was obtained as the major product.

The characterization of both compounds was performed by the usual analytical techniques indicating the presence of one bisphosphonate and two isopropylamine residues. All the data have been carefully compiled in the Supplementary Material (SM). Brought together, the data were in accordance with either general formulae: $[Pt(ipa)_2(BPs\text{-}H)]$ and/or $[Pt(ipa)_2(BPsH)]NO_3$. The core of the platinum cation is clear from the mass spectra, which in both cases showed the molecular formula of B in solution (see figure 1 for complex **1**) where the bisphosphonate might act as a chelating ligand through two oxygen atoms in a bidentate coordination mode.

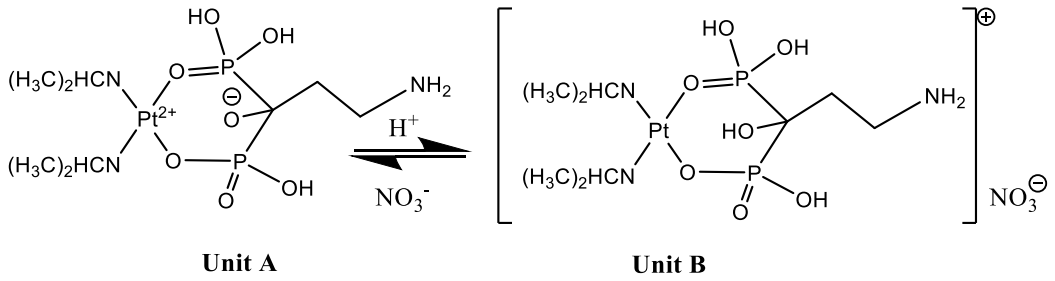

Figure 1. Forms detected for complex **1** by ESI-mass (H_2O) and by NMR in D_2O

The solid state structure of compound **1** (Figure 2) agreed with the formula $[Pt(ipa)_2(BPs)][Pt(ipa)_2(BPsH)]NO_3$. The asymmetric unit $[Pt(ipa)_2(BPsH_{0.5})](NO_3)_{0.5}$ contains only one platinum complex, with 50% occupation for hydroxylic hydrogen atom H7, and half a nitrate anion. A model with a double asymmetric unit was tried but it did not refine well against the experimental data, meaning that that the two species $[Pt(ipa)_2(BPs)]$ and $[Pt(ipa)_2(BPsH)]^+$ are randomly located in the crystal. In the metal complex, there are also two alternative positions for oxygen atoms: O1 to O6 in the phosphonate groups, as well as for the platinum atom, with 75%-25% occupation (see table S3 for hydrogen bond interactions).

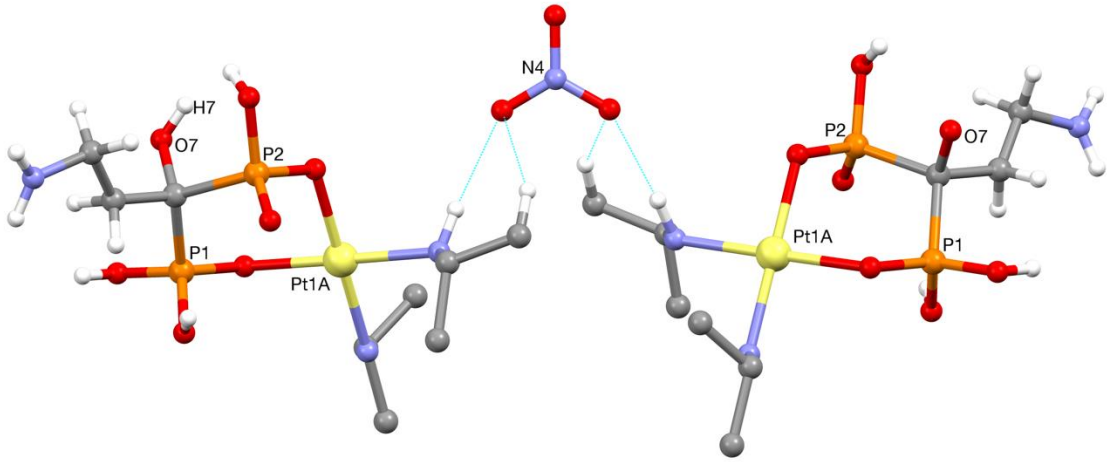

Figure 2. Molecular plot of the $[Pt(ipa)_2(BPs)][Pt(ipa)_2(BPsH)]NO_3$ in **1**, hydrogen interactions are depicted in blue. Hydrogen atoms not participating in hydrogen bonds have been removed for clarity.

Complex **2** was assigned as $[Pt(ipa)_2(ALEN)][Pt(ipa)_2(ALENH)]NO_3$ based in the high similarities in the spectroscopy characterization. The monomeric structures of complexes **1** and **2** are unique in the literature, as the published examples are in most of the cases dinuclear and polynuclear compounds(13,14). The few monomeric examples contained bisphosphonates are N-BPs coordinated to the Pt atom, but in our case the complexes coordinate to platinum just by the O of the bisphosphonate group.

The stability of complexes **1** and **2** in water and Tris-buffer (used to mimic physiological conditions) is presented in Supplementary Figures S1-S2. Solutions of the complexes were tested using UV/Vis spectroscopy at 37°. Both compounds were unusually stable and no hydrolysis of the bisphosphonate was detected monitoring the solution by ^{1}H- and ^{31}P-NMR spectroscopy in D_2O for both complexes (Figure S3). The antiproliferative properties of **1** and **2** were determined on a panel of cancer cell lines (Table 1 and SM)

Table 1 - IC_{50} values for 72 h treatment of four different human cell lines

Compound * Data from literature	IC_{50} (μM)			
	A2780	**A2780cisR**	**MDA MB231**	**PC3**
PAM	59	60.8	134.9	100
1	>200	>200	>200	>200
ALEN	184.4	74.4	69.5	107.6
2	>200	103	100.2	>200
$Pt(ipa)_2I_2$	3.1	5.9	0.4	1.8
Cisplatin	2.3	16.09	3-10	51
Carboplatin *	5-11	4-40	-	-

The free bisphosphonates showed moderate cytotoxicity. Complexes **1** and **2** presented even lower cytotoxicity, with complex **2** being more cytotoxic than complex **1**. Both,complex **2** and its free BP are more potent in the A2780cisR and MDA MB231 cells lines. We also tested cisplatin in the presence of complex **2** in A2780cisR in order to check for a synergistic effect in cell death. However, no increase in cell death was observed versus cisplatin alone.

Complex **1** reaction with CT-DNA was conducted using UV/Vis titration by monitoring the characteristic $\pi \rightarrow \pi^*$ band at 260 nm (Figure 3), typical of B-form of DNA. In these studies, the addition of increasing amounts of complex **1** to a known concentration of CT-DNA (50μM) produced no appreciable changes in the intensity of the DNA absorption. This fact suggests that the complex presents poor CT-DNA affinity.

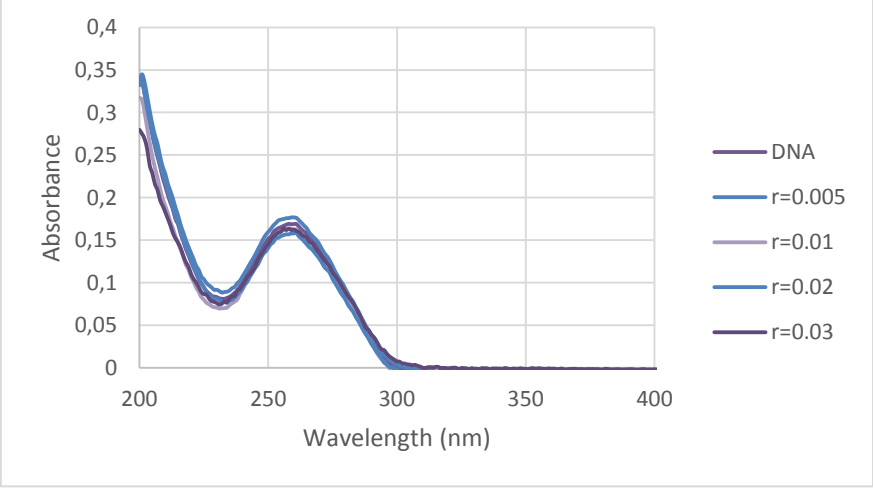

Figure 3. UV absorption spectra of CT-DNA in the presence of increasing amounts of the complex **1** at diverse r_{values}

Cisplatin have been widely reported to produce changes in the mobility of plasmid DNA isoforms in gel electrophoresis,(15), in particular it reduces the supercoiled isoform mobility (via unwinding) and increases the open circular isoform mobility until both reach a co-migration point(16,17). The interaction of complex **1** with pBR322 was evaluated by gel electrophoresis (Figure 4) at different concentrations expressed as r_i (complex **1**: DNA base pairs.). Complex 1 does not alter the electrophoretic mobility of the isoforms of pBR322. From both DNA interaction experiments, we can conclude that the DNA is not a target for this compound, and this might be related to thereduced cytotoxicity.

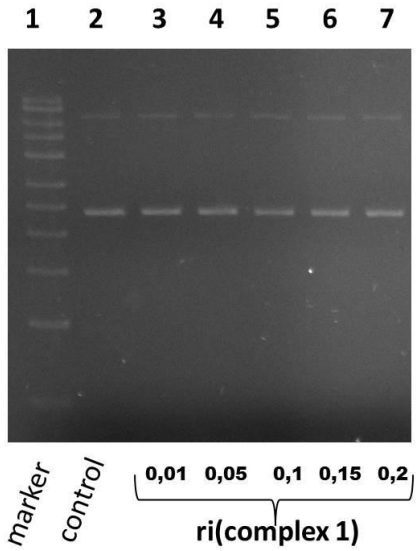

Figure 4. Electrophoresis of plasmid DNA pBR322 after incubation with complex **1** .

In conclusion,complexes **1** and **2** do not show remarkablecytotoxic activity, but their structure confers a good solubility and stability in solution. This indicates the value of the new phosphonate complexes as a robust element in the biological system without a toxicity effect.

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References

1. Palma, E., Correia, J. D. G., Campello, M. P. C., and Santos, I. (2011) *Molecular BioSystems* **7**, 2950-2966
2. Eriksen, E. F., Diez-Perez, A., and Boonen, S. (2014) *Bone* **58**, 126-135
3. Costa, L. (2014) *Current Opinion in Supportive and Palliative Care* **8**, 414-419
4. Costa, L. *The Lancet Oncology* **15**, 15-16
5. Kavanagh, K. L., Guo, K., Dunford, J. E., Wu, X., Knapp, S., Ebetino, F. H., Rogers, M. J., Russell, R. G. G., and Oppermann, U. (2006) *Proceedings of the National Academy of Sciences* **103**, 7829-7834
6. Winter, M. C., Holen, I., and Coleman, R. E. (2008) *Cancer Treat Rev* **34**, 453-475
7. Bloemink, M. J., Keppler, B. K., Zahn, H., Dorenbos, J. P., Heetebrij, R. J., and Reedijk, J. (1994) *Inorg Chem.* **33**, 1127-1132
8. Klenner, T., Wingen, F., Keppler, B., Valenzuela-Paz, P., Amelung, F., and SchmÃ¤hl, D. (1990) *Clinl & Exp. Metastasis* **8**, 345-359
9. Klenner, T., Wingen, F., Keppler, B. K., Krempien, B., and SchmÃ„hl, D. (1990) *J. Cancer Res. Clin.Onc.* **116**, 341-350
10. Galanski, M., Slaby, S., Jakupec, M. A., and Keppler, B. K. (2003) *J Med Chem* **46**, 4946-4951
11. Woynarowski, J. M., Faivre, S., Herzig, M. C. S., Arnett, B., Chapman, W. G., Trevino, A. V., Raymond, E., Chaney, S. G., Vaisman, A., Varchenko, M., and Juniewicz, P. E. (2000) *Mol Pharm* **58**, 920-927
12. Palazzo, B., Iafisco, M., Laforgia, M., Margiotta, N., Natile, G., Bianchi, C. L., Walsh, D., Mann, S., and Roveri, N. (2007) *Adv. Funct Materials* **17**, 2180-2188
13. Margiotta, N., Ostuni, R., Gandin, V., Marzano, C., Piccinonna, S., and Natile, G. (2009) *Dalton Trans.* 10904-10913
14. Margiotta, N., Capitelli, F., Ostuni, R., and Natile, G. (2008) *J. Inorg. Biochem.* **102**, 2078-2086
15. Johnstone, T. C., Suntharalingam, K., and Lippard, S. J. (2016) *Chem. Rev.* **116**, 3436-3486
16. Quiroga, A. G., Perez, J. M., Montero, E. I., Masaguer, J. R., Alonso, C., and Navarro-Ranninger, C. (1998) *J. Inorg. Biochem.* **70**, 117-123
17. Roberts, J. D., Van Houten, B., Qu, Y., and Farrell, N. P. (1989) *Nucleic Acids Res.* **17**, 9719-9733
18. AINT+NT Version 6.04, S. A.-D. I. P. B. A. X.-r. I. M., WI,. (1997−2001) SAX Area-Detector Integration Program; Bruker Analytical X-ray Instruments. Madison, WI,
19. G. M. Sheldrick, S. V., . (1997−2001) Program for Empirical 951 Absorption Correction; . University of Göttingen: Germany
20. 6.10, B. A. S. V. (2000) Structure Determination Package; Bruker Analytical X-ray Instruments. Madison, WI

Anexo II

Technetium-99m complexes of L-arginine derivatives for cancer imaging

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Abstract

Radiotracers targeting cationic amino acid transporters, namely those based on metal complexes, are rather unexplored, despite having relevant potential from the clinical viewpoint,. The rare examples of complexes recognized by amino acid transporters, namely by the Na^+-independent neutral L-type amino acid transporter 1 (LAT1), are ^{99m}Tc(I)/Re(I) compounds. Herein, we describe conjugates comprising a pyrazolyl-diamine chelating unit and the cationic amino acid L-Arg linked by a propyl (**L^1**) or hexyl linker (**L^2**), which allowed the preparation of stable complexes of the type *fac*-[^{99m}Tc(CO)$_3$(κ^3-L)] (**Tc1**, L = **L^1**; **Tc2**, L = **L^2**) and of the respective surrogates **Re1** and **Re2**. Interestingly, complex **Tc2** exhibited moderate levels of time-dependent internalization in three human tumoural cell lines, with approximately 3 % of total applied activity internalized, corresponding to 21 % of cell-associated activity. The surrogate complex **Re2** does not recognize iNOS, a putative mechanism of retention in the cytoplasm of cells, as demonstrated by the in vitro assays with purified iNOS and in studies with LPS-activated macrophages. Preliminary mechanistic studies suggest that the internalization of **Tc2** is linked to the cationic amino acid transporters, namely system y^+. This finding might open the way towards the development of novel families of metal-based radiotracers for probing metabolically active cancer cells.

Introduction

The majority of the Positron Emission Tomography (PET) imaging procedures rely on the use of the glucose analogue 2-[^{18}F]fluoro-2-deoxy-D-glucose ([^{18}F]FDG), which enters the cells via membrane glucose transporters, where it undergoes phosphorylation and is irreversibly trapped. [1, 2] The accumulation of this radiopharmaceutical in tumoural cells is mainly due to the upregulation of glucose transport and glycolysis. However, [^{18}F]FDG presents some limitations, including limited visualization of brain tumors, low orvariable uptake in some tumors types (e.g. prostate cancer and neuroendocrine tumors) and increased accumulation in inflammatory lesions.[3, 4] Thus, radiolabeled amino acids, which target increased rates of amino acid transport in cancer cells, have been considered as alternatives to overcome some of those limitations.[5, 6] Indeed, they are accepted as tracers for imaging the upregulated metabolism linked to several hallmarks of cancer.

The transport of amino acids across the plasma membrane into the cytoplasm of mammalian cells is mediated by membrane-bound transport systems that present varying substrate specificities, pH and sodium dependence, and regulatory mechanisms.[6-8] Most of the work performed so far was based on radiolabeled amino acids for targeting the "L amino acid transport system", which preferentially transports amino acids with neutral side chains like L-Leu, L-Tyr, and L-Phe.[9, 10] Relevant tracers from this class include L-^{11}C-methionine and O-(2-^{18}F-fluorethyl)-L-Tyrosine (^{18}F-FET) for imaging brain tumors, and 6-^{18}F-fluoro-3,4-dihydroxy-L-phenylalanine (^{18}F-FDOPA) for imaging of neuroendocrine tumors. In the past few years there has been a growing interest in the design of tracers for targeting other amino acid transporters, including "system A", glutamine, glutamate and cationic amino acid transporters.[9] The transport of basic amino acids (L-Lys, L-Arg and L-His) is mediated by sodium-independent and sodium-dependent transporter systems, which include the cationic amino acid transporter (CAT, system y^+) family, system y^+L, $b^{o,+}AT$ and $ATB^{0,+}$.

The amino acid L-Arg is the precursor for relevant metabolic pathways such as agmatine, creatine, urea, and nitric oxide (NO) synthesis.[11] The latter is a mammalian signaling molecule biosynthesized by NO synthases (NOS) with high relevance in physiological (e.g. neuronal transmission) and

pathological processes (e.g. cancer and neurological disorders).[12-14] Additionally, L-Arg is a relevant signaling molecule that regulates essential cellular functions such as protein synthesis, apoptosis and growth.[11] This amino acid also plays an important role in cells lacking argininosuccinate synthase 1 (ASS1), one of the key urea cycle enzymes that is absent in many tumors, suggesting that tumoral ASS1 deficiency may be both a prognostic biomarker and predictor of sensitivity to arginine deprivation therapy.[15] Brought together the pathophysiological roles of L-Arg and the fact that certain cancer cells overexpress cationic amino acid transporters such as $ATB^{0,+}$ or CAT1, [16-20] indicate that radiolabelled L-Arg derivatives hold potential for cancer imaging. Moreover, radiolabelled L-Arg derivativescould be envisaged as imaging biomarkers for predicting and monitoring response to arginine deprivation therapy.

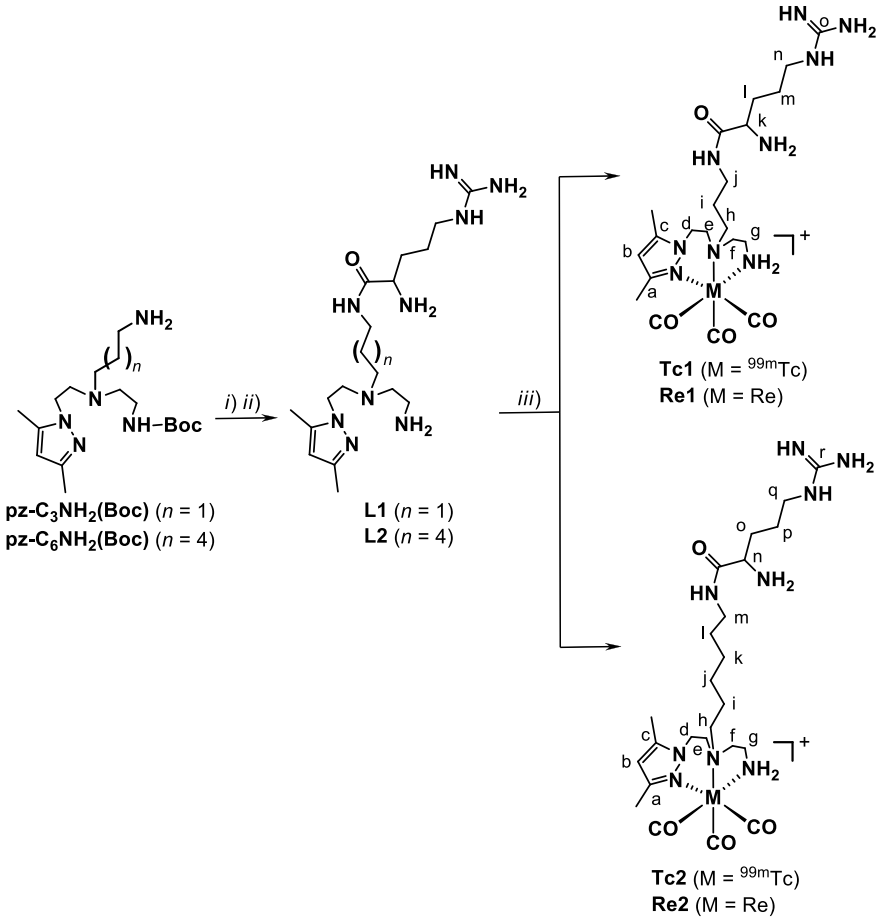
Although potentially relevant from the clinical point of view, radiotracers targeting cationic amino acid transporters are relatively unexplored and, in particular, no metal-based radiotracers are known.[6, 9] So far, the only examples of metal complexes recognized by amino acid transporters and actively internalized into cancer cells, more specifically through the L-type LAT1, are ^{99m}Tc(I)/Re(I) complexes.[21] One of the main advantages of using ^{99m}Tc-based complexes, compared to using cyclotron-produced radionuclides such as ^{11}C or ^{18}F, relies on fact that ^{99m}Tc is affordable, easily available in many clinics worldwide through generators, and burdens a low dose to patients. Considering our previous work in the design and biological evaluation of novel Tc(I)/Re(I) complexes with pendant L-Arg derivatives for visualization of NO/NOS-related tumors by SPECT imaging,[22-26] we describe herein the (radio)synthesis, biological evaluation and preliminary mechanistic studies of novel ^{99m}Tc(I)-labelled L-Arg derivatives useful for imaging metabolically active cells.

Results and discussion

Complexes of the type *fac*-[$M(CO)_3(k^3\text{-}L)$] (M = Re/^{99m}Tc, L = L^1 and L^2)

We have prepared and fully characterized conjugates $\mathbf{L^1}$ and $\mathbf{L^2}$ containing L-Arg derivatives following previously described procedures.[22] Besides the pendant amino acid moiety, $\mathbf{L^1}$ and $\mathbf{L^2}$ present a pyrazoly-diamine chelating unit that is known to stabilize efficiently the organometallic

core *fac*-$[M(CO)_3]^+$ (M = ^{99m}Tc or Re), which already allowed the radiolabeling of various molecules with biological relevance.[23, 27] In brief, the conjugates were prepared upon conjugation of the Boc-protected precursors **Pz-C_3NH_2(Boc)** and **Pz-C_6NH_2(Boc)** to *N*-α-Boc-L-Arg (Scheme 1), respectively, using standard coupling reagents and conditions (HBTU, Et_3N, 2 h, room temperature), followed by hydrolysis of the protecting groups with trifluoroacetic acid (TFA).



Scheme 1. Synthesis of **L^1**, **L^2**, **Tc1**/**Re1** and **Tc2**/**Re2** (*Identification system for NMR assignments is displayed for the rhenium complexes*). i) Et_3N, *N*-α-Boc-L-Arg, HBTU, 2 h, r.t.; ii) CH_2Cl_2-TFA, 3 h, r.t.; iii) $[M(CO)_3(H_2O)_3]^+$ (M = Re, ^{99m}Tc) , H_2O, 100°C.

Conjugates **L^1** and **L^2** were obtained in high purity (> 95%) as stable colorless oils after purification by semi-preparative reversed phase high performance liquid chromatography (RP-HPLC). The conjugates were fully characterized by $^1H/^{13}C$ NMR (including 2D-NMR experiments such as ^1H–^1H correlation spectroscopy, COSY and ^1H–^{13}C heteronuclear single quantum coherence, HSQC) and IR spectroscopy as well as electrospray ionization mass spectrometry (ESI-MS) (Supplementary Figures?)

The radioactive complexes *fac*-[^{99m}Tc(CO)$_3$(k^3-L)] (**Tc1**, L = **L^1**; **Tc2**, L = **L^2**, Scheme 1) were prepared in high radiochemical yield and radiochemical purity (> 95%) upon reaction of **L^1** or **L^2** with the precursor *fac*-[^{99m}Tc(CO)$_3$(H$_2$O)$_3$]$^+$. The latter was prepared by addition of Na[^{99m}TcO$_4$], eluted from a ^{99}Mo/^{99m}Tc generator with saline solution, to an IsoLink kit (Mallinckrodt, Covidien) available for research purposes, and heating (95 °C) for 20 min. The high stability of the resulting final complexes was demonstrated by incubation with a 100-fold excess of coordinating amino acids such as histidine or cysteine. No degradation or transchelation were detected by RP-HPLC after incubation at 37°C for up to 6h (Supplementary Figures??). , in line with earlier results obtained for complexes stabilized by the same chelating unit.[22, 27, 28] Additional stability studies demonstrated that the complexes are also stable in human plasma.

The nature of solutions of ^{99m}Tc complexes (ca. 10^{-9} - 10^{-12} M) hampers their structural characterization by the usual analytical methods in chemistry. The more straightforward way to overcome this limitation is to compare the chromatographic behavior of ^{99m}Tc complexes with that of the surrogate rhenium complexes prepared at the "macroscopic" scale, since technetium and rhenium which are both transition metals of group 7 of the periodic table, share similar coordination chemistry. Thus, the chemical identity of **Tc1** and **Tc2** was confirmed by comparing their RP-HPLC radioactive traces (γ detection) with the UV-Vis traces of the surrogate complexes **Re1** and **Re2. These complexes** were synthesized upon reaction of **L^1** and **L^2** with *fac*-Re(CO)$_3$(H$_2$O)$_3$]$^+$ in refluxing water (Scheme 1), and were obtained in moderate yields (35 – 75%) after purification by semi-preparative RP-HPLC (> 95% purity).

Re1 and **Re2** were fully characterized by ESI-MS, IR and NMR spectroscopy (^{1}H/^{13}C NMR, ^{1}H-^{1}H COSY, and ^{1}H-^{13}C HSQC). The data collected support the proposed structure and the tridentate coordination mode of the pyrazolyl-diamine chelating unit, comparing well with similar complexes previously described by our group[22, 27, 29].

Cellular uptake studies

Aimed at predicting the *in vivo* tumor-targeting properties of **Tc1** and **Tc2** and to assess their ability to be recognized and internalized as specific substrates by the transporters of L-Arg, we have performed uptake studies in a panel of human tumoural cell lines, more specifically Hela cervical cancer cell line, A375 melanoma cell line, MDA-MB-231 breast cancer cell line and PC3 prostate cancer cell line. The results of the cellular uptake as a function of the incubation time are presented in Figure 1.

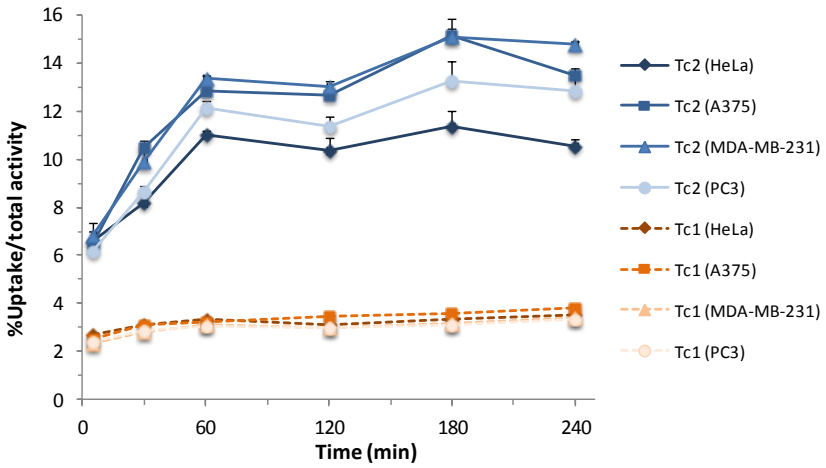
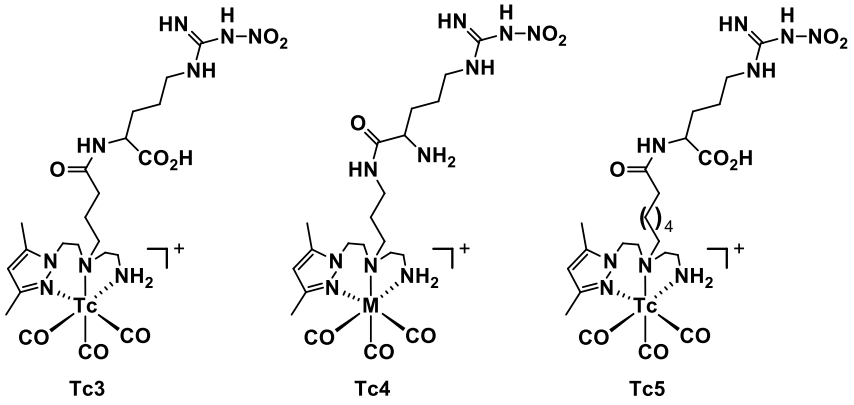


Figure 1. Cellular uptake of **Tc1** and **Tc2** in human cancer cell lines at 37°C.

Complex **Tc2** displayed a remarkably higher uptake than **Tc1**, which values between 11.4±0.7 % (Hela cell line) and 15.1±0.3 % (A375 cell line) after 3 h incubation. We speculate that the difference observed between the uptake values for the two radioconjugates might be due to the presence of a longer alkyl chain in **Tc2** (hexyl linker) than in **Tc1** (propyl linker), assuming that these arginine derivatives are substrates of the Na^+-independent transport system y^+, which has been postulated to be the major entry route for cationic amino acids, L-Arg included, in most cells.

Indeed, it has been reported that for system y^+, CAT proteins have an higher affinity for cationic amino acids with a long carbon backbone: homoarginine > arginine > lysine > ornithine > 2,4-diamino-*n*-butyric acid.[8] Other clue suggesting the involvement of the system y^+ in the cellular

uptake of **Tc1** and **Tc2** is the fact that analogue radioconjugates with pendant N^{ω}-NO_2-L-arginine moieties (**Tc3** - **Tc5**, Scheme 2), previously prepared for targeting NOS,[22, 28] are not taken up by the cell lines tested (Figure 2).



Scheme 2. Molecular structures of **Tc3** - **Tc5**.

In fact, it has been described in the literature that inhibitors of NOS such as N^{ω}-NO_2-L-arginine methyl ester or N-methyl arginine competitively inhibit arginine transport across cell membranes due to interaction with system y^+.[30]

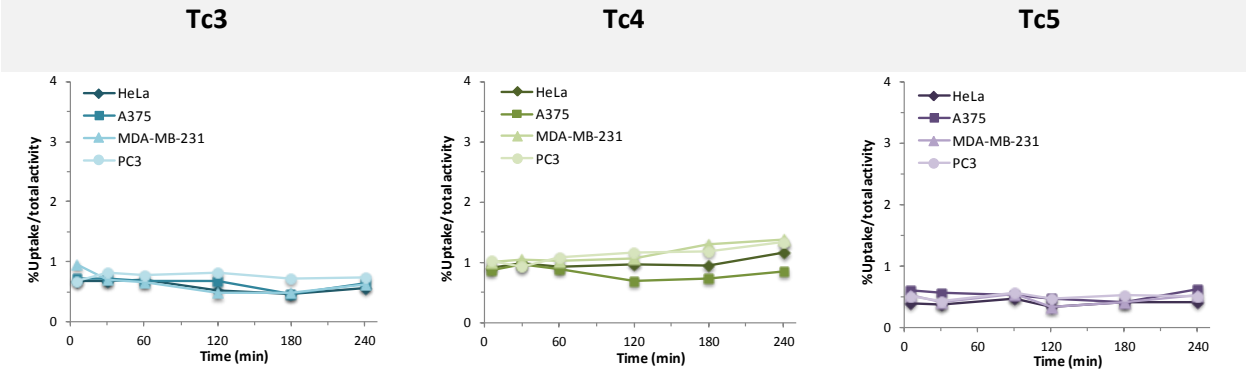
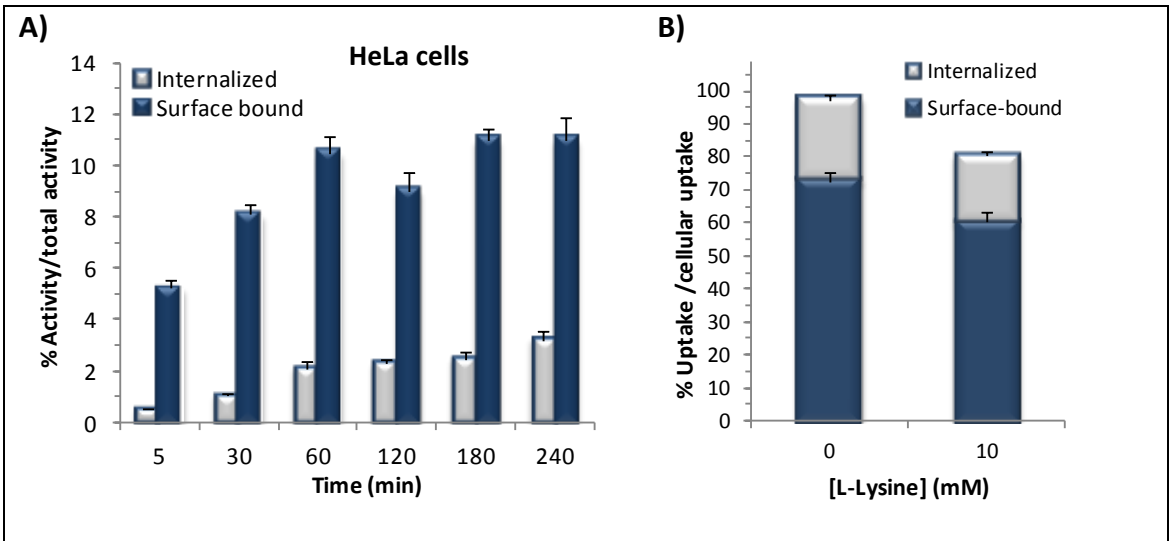


Figure 2. Cellular uptake of the radioconjugates **Tc3 – Tc5** in various human cancer cell lines at 37°C.

The remarkable cellular uptake results obtained with **Tc2** prompted us to deepen these studies to attempt to unveil the most likely cellular uptake mechanism, using both the radioactive complex

Tc2 and the "cold" surrogate **Re2**. Therefore, internalization studies in HeLa, A375 and MDA-MB-231 cancer cell lines have been performed, and the results are presented in Figure 3A.



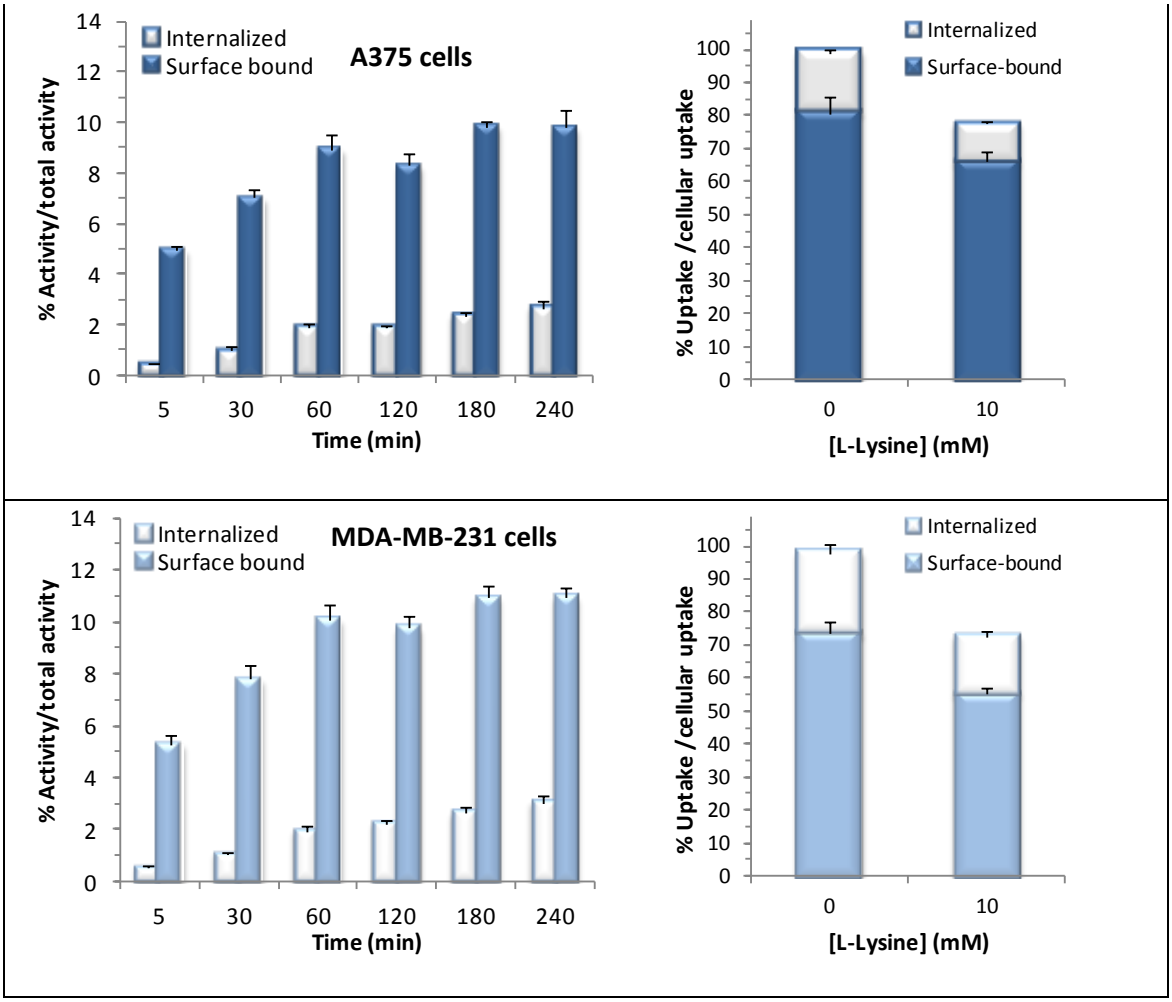


Figure 3. A) Internalized and surface-bound **Tc2** in HeLa, A375 and MDA-MB-231 cancer cell lines at different time points at 37°C, expressed as a percentage of total applied ?? activity. **B)** Internalized and surface-bound **Tc2** after 2 h in the presence of lysine at 37°C, expressed as a percentage of cellular uptake in the absence of L-Lys.

Tc2 exhibits moderate levels of time-dependent internalization with the highest values being reached at 4 h in all cell lines: 2.7 ± 0.2 % (A375), 3.0 ± 0.3 % (MDA-MB-231) and 3.2 ± 0.3 % (HeLa) of the total applied activity internalized, corresponding to 21.3 ± 0.8 %, 21.6 ± 1.3 % and 22.5 ± 1.0 % of the cell-associated activity, respectively.

Aiming to evaluate the specificity of **Tc2** cellular uptake, namely the contribution of the cationic amino acid transporter more frequently associated to cancer cells, CAT1 (system y^+), we have performed internalization studies of **Tc2** in the same cell lines at 2 h (37 °C) with co-incubation with

lysine (Figure 3B), whose transport across cell membranes is mediated by the CAT family. The expression of CAT1 in the selected cell lines was confirmed by Western blot analysis of protein extracts using an anti-CAT1 antibody (Figure 4).

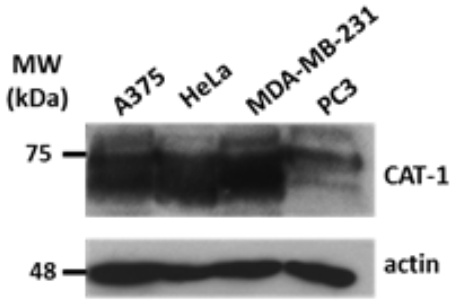


Figure 4. Evaluation of CAT1 expression in HeLa cervical cancer cell line, A375 melanoma cell line, MDA-MB-231 breast cancer cell line and PC3 prostate cancer cell line. Actin was used as an internal loading control.

Although moderate, the inhibition of cell surface-bound **Tc2** (17 %, 25 % and 18% for HeLa, MDA-MB-231 and A375 cell lines, respectively) and more importantly the inhibition of internalized **Tc2** (20 %, 29 % and 36 %, respectively) (Figure 3B) suggests that the internalization of **Tc2** is partially mediated by system y^+.

Additionally, we have also performed the cell uptake assays in the presence of N-ethylmaleimide (NEM), a specific inhibitor of system y^+. The results are presented in the Figure 5.

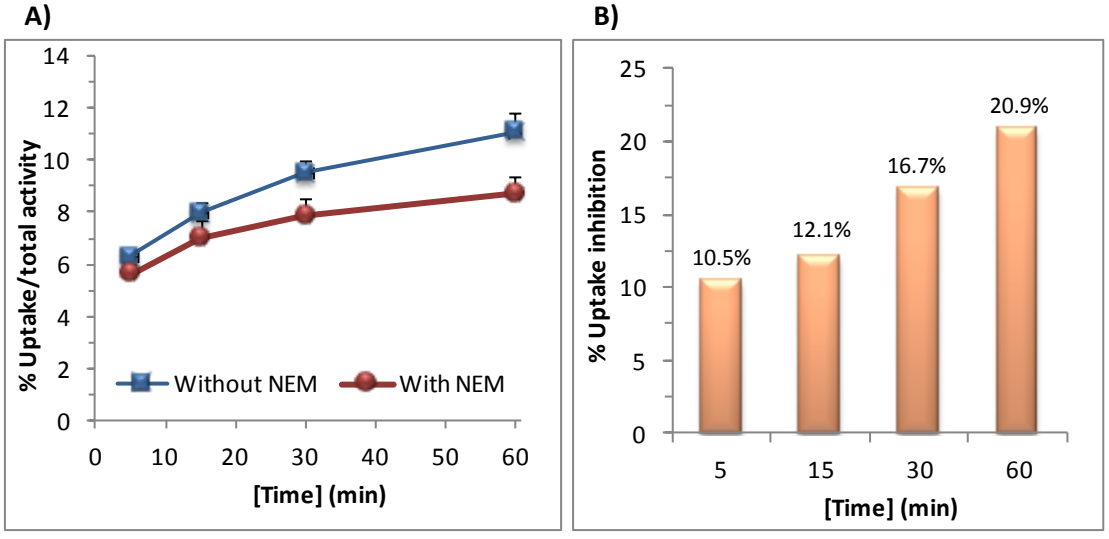


Figure 5. **A)** Cellular uptake of **Tc2** in the A375 melanoma cell line at different time points at 37°C in the presence of N-ethylmaleimide (NEM, 5 mM). **B)** Inhibition of cellular uptake by NEM expressed in percentage.

Under the conditions tested, the cellular uptake of **Tc2** is inhibited by NEM in 10.5 %-20.9 % after 5 to 60 minutes of incubation (Figure 5B), which supports our hypothesis of CAT1-mediated internalization of this complex.

Although the results presented above may suggest that the sodium-independent transporter CAT1 is implicated in the transport of **Tc2** across cell membranes, the contribution of sodium-dependent transporters such as amino acid transport systems $b^{0,+}AT$ and $ATB^{0,+}$ cannot be discarded at this point.

The driving force for **Tc2** internalization could also be strengthened by the simultaneous contribution of a specific mechanism of retention in the cytosol of the cell, namely by interaction of the complex with cytosolic NOS, which we have already demonstrated to be case for complexes of a related family. To test this hypothesis we have studied the ability of **Re1** and **Re2**, cold surrogates of **Tc1** and **Tc2**, to be recognized by NOS both in enzymatic assays with purified iNOS and in LPS-activated macrophages. For the sake of comparison, the corresponding ligands **L1** and **L2** were also tested.

Firstly, the molecules were tested as NO-producing substrates using mouse recombinant iNOS. The iNOS activity was determined spectrophotometrically by monitoring the NO-mediated conversion of oxyhemoglobin to methemoglobin at 401 nm and 421 nm as previously described.[22, 29] Table 1 displays the kinetic parameter K_m for all compounds, which was determined by the method of Eisenthal and Cornish-Bowden.

Table 1. K_m values for L-Arg, **L1**, **L2**, **Re1** and **Re2**

Compound	K_m values/μM
L-Arg	3.00 ± 1.00
L1	50
Re1	1093
L2	1200
Re2	> 2500

The ability of **L1** (K_m = 50 μM) to interact specifically with the active site of iNOS, leading to NO production, is considerably higher than that observed for **L2** (K_m = 1200 μM) however, both compounds are poorer substrates than the endogenous substrate of the enzyme (K_m = 3 μM). Metallation of **L1** and **L2** led to complexes with even lower NO-producing properties, as evidenced by the determined K_m values (**Re1**, K_m = 1093 μM; **Re2**, K_m > 2500 μM).

The effect of the same compounds was also studied in RAW 264.7 macrophages after treatment with lipopolysaccharide (LPS), which leads to increased NO biosynthesis due to iNOS overexpression.[22, 31] This cellular model is very useful, as it allows to assess both the ability of the compounds to cross cellular membranes, a key feature for improving tracer uptake *in vivo*, and the intracellular interaction with iNOS enzyme via the quantitation of the NO release.

The results, which represent the ability of the compounds to be recognized and used as NOS substrates and, consequently, their efficacy in NO biosynthesis in LPS-activated macrophages cultured in arginine-free medium, are presented in Figure 6.

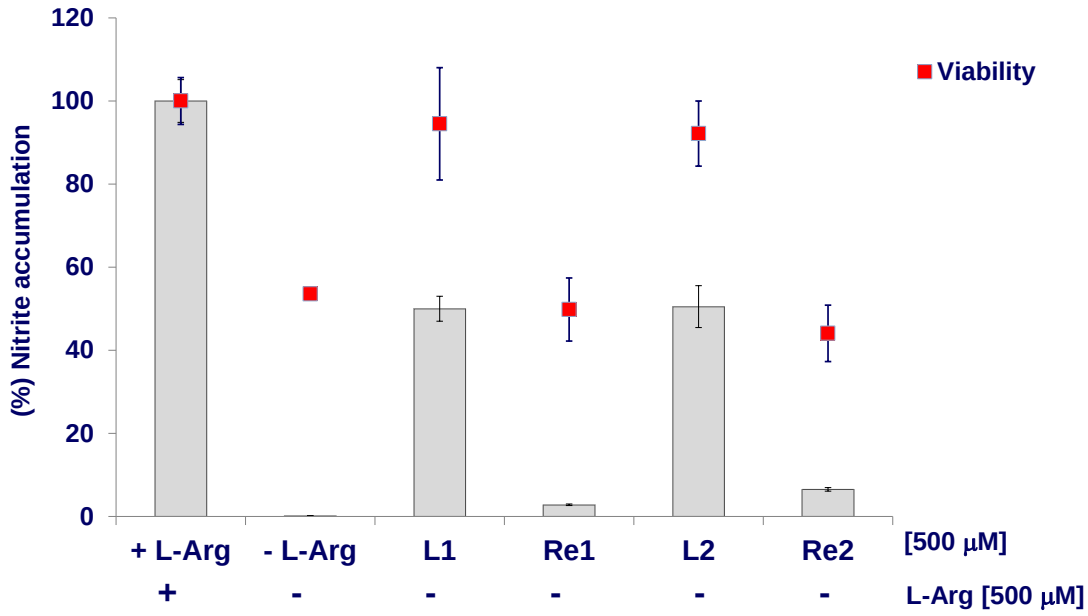


Figure 6: Effect of **L1**, **L2**, **Re1** and **Re2** on NO biosynthesis by LPS-induced RAW 264.7 macrophages. Data are expressed as % of nitrite accumulation of the L-Arg control (mean ± S.D. -standard deviation, n = 8). The experiment was repeated three times with comparable results.

When LPS-induced macrophages were incubated in L-Arg-free culture medium, negligible NO production was observed. However, when macrophages were incubated with various concentrations of L-Arg (100 – 700 μM) a high nitrite accumulation in the extracellular medium was observed (data not shown).

Incubation of LPS-treated macrophages with the new compounds showed that $\mathbf{L^1}$ and $\mathbf{L^2}$ are recognized as substrates by the enzyme, confirmed by NO production (ca. 50%). When the corresponding rhenium compounds **Re1** and **Re2** are incubated with the cells the production of NO was negligible. These results are consistent with those obtained in the enzymatic assays, where it has been possible to conclude that metallation of conjugates **L1** and **L2** led to decreased affinities towards the enzyme.

We also performed a parallel viability assay to assess the intrinsic cytotoxicity of the compounds at the concentration used in the NO assay (500 μM). The compounds were tested in the presence or absence of L-Arg (**Figure 6**) in order to evaluate if the viability is related with the presence/absence of NO, a key signaling mediator in several metabolic processes.

In the case of **L[1]** and **L[2]** the viability is similar in both conditions (approximately 100%). For the metal complexes **Re1** and **Re2**, which are not utilized as substrates, the viability is decreased in the absence of L-Arg, but is comparable to the control when that amino acid is present in the culture medium (results not presented), showing that the compounds by themselves are not toxic at the concentrations tested. All together, these results indicated that viability was being determined by NO biosynthesis.

Conclusions

We have prepared and characterized new conjugates comprising a pyrazolyl-diamine chelating unit and a pendant L-Arg moiety linked by a propyl (**L[1]**) or a hexyl linker (**L[2]**). The conjugates reacted with the organometallic precursors *fac*-$[M(CO)_3(H_2O)_3]^+$ (M = ^{99m}Tc/Re) yielding the radioactive complexes of the type *fac*-$[^{99m}Tc(CO)_3(\kappa^3\text{-L})]$ (**Tc1**, L = **L[1]**; **Tc2**, L = **L[2]**) and the respective "cold" surrogates **Re1** and **Re2**. **Tc2** exhibited moderate levels of time-dependent internalization in three different human tumoural cell models, with about 3 % of the applied activity internalized after 4h at 37 °C, corresponding to 21 % of the total cell-associated activity. Preliminary mechanistic studies suggest that internalization of **Tc2** is mediated by cationic amino acid transporters, namely system y^+, although more extensive amino acid transport assays will be needed to fully address that issue in the cancer lines studied. In addition, enzymatic assays with purified iNOS and studies with LPS-activated macrophages demonstrate that the surrogate complex **Re2**does not recognize a putative target of **Tc2** in the cytosol. Nevertheless, **Tc2** is a rare example of a metal complex whose entrance into cells seems to be mediated by cationic amino acid transporters. This finding might open the way towards the development of novel families of metal-based radiotracers to probe metabolically active cancer cells.

Experimental Section

General procedures and materials: All chemicals and solvents were of reagent grade and were used without purification unless stated otherwise. The BOC-protected precursor *tert*-butyl 2-((3-aminopropyl) (2-(3,5-dimethyl-1H-pyrazol-1-yl)ethyl) amino) ethylcarbamate (**Pz-C_3NH_2(Boc)**), tert-butyl 2-((6-aminohexyl)(2-(3,5-dimethyl-1H-pyrazol-1-yl)ethyl)amino)ethylcarbamate (**Pz-C_6NH_2(Boc)**), $[Re(CO)_3(H_2O)_3]Br$ were prepared according to published methods.[22, 32] 2-(*tert*-Butoxycarbonylamino)-5-guanidinopentanoic acid (*N*-α-Boc-L-Arg) was purchased from Sigma Aldrich as well as all other chemicals not specified above. $Na[^{99m}TcO_4]$ was eluted from a $^{99}Mo/^{99m}Tc$ generator, using 0.9 % saline. The radioactive precursor *fac*-$[^{99m}Tc(CO)_3(H_2O)_3]^+$ was prepared using a IsoLink® kit (Covidean Malinckrodt, Inc.). ^1H and ^{13}C NMR spectra were recorded at room temperature on a Varian Unity 300 MHz spectrometer. ^1H and ^{13}C chemical shifts were referenced with the residual solvent resonances relatively to tetramethylsilane. The spectra were assigned with the help of 2D experiments (^1H–^1H correlation spectroscopy, COSY and ^1H–^{13}C heteronuclear single quantum coherence, HSQC). Assignments of the ^1H and ^{13}C NMR resonances are given in accordance with the identification system shown in Scheme 1. Infrared spectra were recorded as KBr pellets on a Bruker Tensor 27 spectrometer. All compounds were characterized by electrospray ionization mass spectrometry (ESI-MS) using a Bruker model Esquire 3000 plus. HPLC analyses were performed on a Perkin Elmer LC pump 200 coupled to a Shimadzu SPD 10AV UV/Vis and to a Berthold-LB 509 radiometric detector, using an analytic Macherey-Nagel C18 reversed-phase column (Nucleosil 100-5, 250 x 3 mm) with a flow rate of 0.5 mL/min.

Purification of the inactive compounds was achieved on a semi-preparative Macherey-Nagel C18 reversed-phase column (Nucleosil 100-7, 250 x 8 mm) or on a preparative Waters µBondapak C18 (150 x 19 mm) with a flow rate of 2.0 mL/min and 5.5 mL/min, respectively. UV

detection: 220 or 254 nm. Eluents: aqueous 0.1 % CF_3CO_2H/MeOH. Gradient: t = 0-5 min: 10 % MeOH; 5-30 min: 10→100 % MeOH; 30-34 min: 100 % MeOH; 34-35 min: 100→10 % MeOH; 35-40 min: 10 % MeOH.

Aliquots of ~ 5 mg of pure compounds (≥ 98 % ascertained by RP-HPLC) were lyophilized in microcentrifuge tubes and used for radioactive labelling and *in vitro* studies.

Synthesis of *tert*-butyl 2-((3-(2-amino-5-guanidinopentanamido)propyl)(2-(3,5-dimethyl-1H-pyrazol-1-yl)ethyl)amino)ethylcarbamate (L^1-Boc)

To a solution of **Pz-C_3NH_2(Boc)** (0.050 g, 0.140 mmol) in dimethylformamide (DMF) were added triethylamine (0.036 g, 0.365 mmol) and O-benzotriazol-1-yl-N,N,N´,N´-tetramethyluronium hexafluorophosphate (HBTU, 0.054 g, 0.145 mmol). After 10 min, N-α-Boc-L-Arg (0.039 g, 0.142 mmol) was added, and the reaction mixture stirred at room temperature under a nitrogen atmosphere for 2 h. The solvent was removed under vacuum, and the residue purified by silica gel column chromatography using a gradient of MeOH (0 → 100 %) in $CHCl_3$. The intermediate **L^1-Boc** was obtained as a yellowish oil. Yield: 58.1 % (0.050 g, 0.082 mmol).

^{1}H-NMR (300 MHz, $CDCl_3$): δ_H (ppm) 7.69 (2H, br s, NH), 6.48 (1H, br s, NH), 5.70 (1H, s, CH^b), 5.10 (1H, br s, NH), 5.01 (2H, t, CH_2^d), 4.57 (1H, br s, NH), 3.35 (2H, t, CH_2^e), 3.07 (1H, br m, CH^k), 2.86-2.80 (4H, m, $CH_2^{g,j}$), 2.35 (2H, t, CH_2^n), 2.32 (2H, t, CH_2^f), 2.23 (2H, t, CH_2^g), 2.19 (3H, s, CH_3^{Pz}), 2.16 (3H, s, CH_3^{Pz}), 1.40-1.38 (18H, s, CH_3^{Boc}), 1.39 – 1.15 (6H, m, $CH_2^{i,l,m}$).

^{13}C-NMR (75.5 MHz, $CDCl_3$): δc (ppm) 174.3 (CO), 172.6 (CO), 159.4 (C^o), 147.3 (C^{Pz}), 143.9 (C^{Pz}), 106.9 (C^{Pz}), 79.9 ($C(CH_3)_3$), 56.7 (C^e), 54.1 (C^k), 53.8 (C^f), 51.6 (C^h), 49.2 (C^d), 41.2 (C^n), 38.9 (C^g), 38.0 (C^j), 30.8 (C^i), 28.8 ($C(CH_3)_3$), 25.4 (C^l), 24.6 (C^m), 13.4 (CH_3Pz), 11.0 (CH_3Pz).

Synthesis of *tert*-butyl 2-((6-(2-amino-5-guanidinopentanamido)hexyl)(2-(3,5-dimethyl-1H-pyrazol-1-yl)ethyl)amino)ethylcarbamate (L^2-Boc)

To a solution of **Pz-C_6NH_2(Boc)** (0.050 g, 0.131 mmol) in DMF were added triethylamine (0.036 g, 0.365 mmol) and O-benzotriazol-1-yl-N,N,N´,N´-tetramethyluronium hexafluorophosphate (HBTU,

0.054 g, 0.145 mmol). After 10 minutes, N-α-Boc-L-Arg (0.039 g, 0.142 mmol) was added, and the reaction mixture stirred at room temperature under a nitrogen atmosphere for 2 h. The solvent was then removed under vacuum, and the residue purified by silica gel column chromatography using a gradient of MeOH (0 → 100 %) in $CHCl_3$. The intermediate **L2-Boc** was obtained as a yellowish oil. Yield: 60.2 % (0.050 g, 0.078 mmol).

^{1}H-NMR (300 MHz, $CDCl_3$): δ_H (ppm) 7.70 (2H, br s, NH), 6.51 (1H, br s, NH), 5.73 (1H, s, CH^b), 5.14 (1H, br s, NH), 5.05 (2H, t, CH_2^d), 4.61 (1H, br s, NH), 3.37 (2H, t, CH_2^e), 3.13 (1H, br m, CH^n), 3.13-3.06 (4H, m, $CH_2^{g,m}$), 2.65 (2H, t, CH_2^h), 2.52 (2H, t, CH_2^q), 2.43 (2H, t, CH_2^f), 2.20 (3H, s, CH_3^{Pz}), 2.18 (3H, s, CH_3^{Pz}), 1.80 (2H, q,C^o), 1.80 (2H, m,C^l), 1.42-1.40 (18H, s, CH_3^{Boc}), 1.32 – 1.26 (6H, m, $CH_2^{i,j,k}$).

^{13}C-NMR (75.5 MHz, $CDCl_3$): δc (ppm) 174.5 (CO), 172.8 (CO), 158.4 (C^r), 147.5 (C^{Pz}), 144.2 (C^{Pz}), 105.9 (C^{Pz}), 79.5 ($C(CH_3)_3$), 56.7 (C^e), 56.3 (C^h), 54.1 (C^f), 53.6 (C^n), 49.4 (C^d), 41.5 (C^q), 39.3 (C^m), 38.8 (C^g), 30.6 (C^n), 28.9 ($C(CH_3)_3$), 28.3 (C^i), 27.6 (C^j), 26.4 (C^k), 25.6 (C^o), 24.9 (C^p), 13.5 (CH_3^{Pz}), 11.3 (CH_3^{Pz}).

Synthesis of 2-amino-N-(3-((2-aminoethyl)(2-(3,5-dimethyl-1H-pyrazol-1-yl)ethyl)amino)-propyl)-5-guanidinopentanamide (L^1)

Compound L^1 was obtained directly by dissolving L^1**-Boc** (0.050 g, 0.082 mmol) in a mixture CH_2Cl_2–TFA (1 mL – 3 mL) and allowed to react for 3 h at room temperature with stirring. The residue obtained after evaporation of the solvents was dissolved in water, filtered through a 0.45 μm Millipore filter, and purified by preparative RP-HPLC. The fractions containing L^1 were collected and the solvent removed to provide a clear viscous oil. Yield: 78 % (0.026 g, 0.061 mmol, calcd. for $C_{19}H_{41}N_9O$).

^{1}H-NMR (300 MHz, $CDCl_3$): δ_H (ppm) 5.99 (1H, s, H^b), 4.37 (2H, s, H^d), 3.86 (1H, t, H^n), 3.49 (2H, m, H^e), 3.39 (2H, m, H^f), 3.32 (2H, t, H^g), 3.24-3.06 (6H, t, $H^{q, h, j}$) 2.19 (3H, s, CH_3^{Pz}), 2.10 (3H, s, CH_3^{Pz}), 1.81 (4H, m, $H^{o, i}$), 1.53 (2H, m, H^p).

^{13}C-NMR (75.5 MHz, $CDCl_3$): δc (ppm) 169.9 (CO), 156.9 (C^r), 148.9 (C^c), 144.4 (C^a), 107.3 (C^b), 52.9 (C^n), 51.8 (C^h), 51.7 (C^e) 49.9 (C^f), 42.2 (C^d), 40.4 (C^q), 36.4 (C^j), 33.9 (C^g), 28.1 (C^i), 23.8 (C^o), 23.4 (C^p), 11.5 (CH_3^{Pz}), 10.1(CH_3^{Pz}).

RP-HPLC (t_R): 18.7 min.

ESI-MS (+) (m/z): 412.7 $[M + H]^+$, calcd. for $C_{19}H_{41}N_9O$ = 411.6.

IR (KBr, cm^{-1}): 3445 M ν(NH_2, NH); 1612 S ν(C=O), δ(NH_2), δ(NH, amide), 1480 w ν(CN, amide); 1220 e 1137 S ν(C-N); 909 w, 836 w, 765 w.

Synthesis of 2-amino-N-(6-((2-aminoethyl)(2-(3,5-dimethyl-1H-pyrazol-1-yl)ethyl)amino)-hexyl)-5-guanidinopentanamide (L^2)

Removal of Boc protecting group of **L^2-Boc** was done following the methodology described for **L^1-Boc**. The residue obtained after evaporation of the solvents was dissolved in water, filtered through a 0.45 µm Millipore filter, and purified by preparative RP-HPLC. The fractions containing **L^2** were collected and the solvent removed to provide an yellow viscous oil. Yield: 90.4 % (0.019 g, 0.043 mmol, calcd. for $C_{21}H_{43}N_9O$).

^{1}H-NMR (300 MHz, $CDCl_3$): δ_H (ppm) 6.02 (1H, s, H^b), 4.42 (2H, t,H^d), 3.83 (1H, t, H^n), 3.69 (2H, t, H^e), 3.51 (2H, m, H^f), 3.35 (2H, m, $H^{g', g''}$), 3.13 (6H, m, $H^{q, h, m}$), 2.21 (3H, t, CH_3^{Pz}), 2.13 (3H, s, CH_3^{Pz}), 1.78 (2H, m, H^o) 1.55-1.49 (4H, m, $H^{i, p}$), 1.42-1.32 (2H, m, H^l), 1.22 (4H, m, $H^{j, k}$).

^{13}C-NMR (75.5 MHz, $CDCl_3$): δc (ppm) 169.6 (CO), 156.8 (C^r), 148.2 (C^{Pz}), 145.8 (C^{Pz}), 107.9 (C^b), 53.9 (C^q), 52.9 (C^n), 51.0 (C^e), 49.7 (C^f), 42.0 (C^d), 40.3 (C^m), 39.4 (C^h), 33.6 (C^g), 28.0 ($C^{o, l}$), 25.6 (C^p), 25.2 (C^i), 23.7 (C^k), 22.8 (C^j), 10.9 (CH_3^{Pz}), 10.1(CH_3^{Pz}).

RP-HPLC (t_R): 19.5 min.

ESI-MS (+) (m/z): 220 $[M + H]^{2+}$, 439 $[M+H]^+$, calcd. for $C_{21}H_{43}N_9O$ = 438.

IR (KBr, cm^{-1}): 3312 M ν(NH_2, NH); 1671 S ν(C=O), δ(NH_2), δ(NH, amide), 1421 w ν(CN, amide); 1209 and 1190 S ν(C-N); 898 w, 823 w, 748 w.

General procedure for the preparation of the Re complexes *fac*-[Re(CO)$_3$(k^3-L)] (Re1, L = L1; Re2, L = L2)

$[Re(CO)_3(H_2O)_3]Br$ was reacted with equimolar amounts of **L^1** or **L^2** in refluxing water for 12 h. The solvent was concentrated to ¼ of the volume and the resulting solution was centrifuged and the supernatant purified by preparative RP-HPLC.

Synthesis of *fac*-[Re(CO)$_3$(k^3-L^1)]$^+$ (Re1): Starting from 0.020 g (0.048 mmol) of **L^1**, a colorless oil formulated as **Re1** was obtained. Yield: 33.3 % (0.010 g, 0.014 mmol, calcd. for $C_{22}H_{41}N_9O_4Re$).

^{1}H-NMR (300 MHz, $CDCl_3$): δ_H (ppm) 6.08 (1H, s, H^b), 5.10 (1H, s, NH), 4.35 (1H, dd, $H^{d'}$), 4.14-3.98 (1H, m, $H^{d''}$), 3.87 (1H, t, H^n), 3.71-3.66 (2H, m, NH), 3.59-3.50 (1H, m, $CH^{h'}$), 3.40-3.19 (4H, m, $H^{h'', e', j}_2$), 3.11 (3H, m, $H^{g', q}_2$), 2.75 (2H, m, H^f), 2.67-2.63 (1H, m, $H^{e''}$), 2.50-2.39 (1H, m, $H^{g''}$), 2.30 (3H, s, CH_3^{Pz}), 2.19 (3H, s, CH_3^{Pz}), 2.10-1.91 (1H, m, $H^{i'}$), 1.86-1.75 (3H, m, $H^{i'', o}_2$), 1.61-1.50 (2H, m, H^p_2).

^{13}C-NMR (75.5 MHz, $CDCl_3$): δc (ppm) 196.1, 194.9, 194.7 (CO, Re), 171.6 (C=O), 164.7 (C^r), 155.7 (C^{Pz}), 146.2 (C^{Pz}), 109.8 (C^{Pz}), 66.7 (C^h), 63.7 (C^f), 55.1 (C^n) 55.4 (C^e), 49.0 (C^d), 44.1 (C^g), 42.4 (C^j), 39.3 (C^q), 30.1 (C^o), 26.2 (C^i), 25.8 (C^p), 17.2 (CH_3^{Pz}), 12.8 (CH_3^{Pz}).

RP-HPLC (t_R): 25.1 min

ESI-MS (+) (m/z): 342 $[M+2H]^{2+}$, calcd. for $C_{21}H_{37}N_9O_4Re$ = 681.3.

IR (KBr, cm^{-1}): 3276 M ν(NH_2 NH); 2026, 1915 S (CO); 1676 S (C=O), δ(NH_2), 1208 – 1135 M ν(CN); 876 w; 758 w; 683 w.

Synthesis of fac-[Re(CO)$_3$(k^3-L^2)]$^+$ (Re2): Starting from 0.020 g (0.045 mmol) of **L^2**, a colorless oil formulated as **Re^2** was obtained. Yield: 37.5 % (0.012 g, 0.017 mmol, calcd. for $C_{24}H_{43}N_9O_4Re$).

^{1}H-NMR (300 MHz, $CDCl_3$): δ_H (ppm) 6.05 (1H, s, H^b), 5.04 (1H, s, NH), 4.32 (1H, dd, $H^{d'}$), 4.08 (1H, dd, $H^{d''}$), 3.82 (1H, t, H^n), 3.61 (2H, s, NH), 3.50-3.40 (1H, m, $H^{h'}$), 3.30-3.18 (3H, m, $H^{h'', m}{}_2$), 3.07 (3H, m, $H^{e', g', q}{}_2$), 2.70 (2H, m, $H^f{}_2$), 2.53 (1H, m, $H^{e''}$), 2.40 (1H, m, $H^{g''}$), 2.27 (3H, s, H^{Pz},), 2.16 (3H, s, H^{Pz}), 1.76 (3H, m, $H^{o', o'', i''}$), 1.69-1.58 (1H, m, $H^{i''}$), 1.49 (4H, m, $H^p{}_2{}', ^l{}_2$) 1.61-1.50 (2H, m, $H^j{}_2{}', ^k{}_2$).

^{13}C-NMR (75.5 MHz, $CDCl_3$): δc (ppm) 194.7, 194.4, 193.0 (CO, Re); 169.2 (C=O), 156.9 (C^r), 153.7 (C^{Pz}), 144.2 (C^{Pz}), 107.9 (C^{Pz}), 67.6 (C^h), 61.9 (C^f) 53.3 (C^e), 53.3 (C^n), 47.0 (C^d), 42.2 (C^g), 40.5 (C^m), 39.6 (C^q), 28.2 (C^l), 28.2 (C^o), 25.9 ($C^{j, K}$), 24.4 (C^i), 23.8 (C^l), 15.3 ($CH_3{}^{Pz}$), 10.9 ($CH_3{}^{Pz}$).

RP-HPLC (t_R): 25.5 min.

ESI-MS (+) (m/z): 355 $[M + 2H]^{2+}$, calcd. for $C_{24}H_{43}N_9O_4Re$ = 708.

IR (KBr, cm^{-1}): 3274 M v(NH_2 NH); 2028, 1914 S (CO); 1675 S (C=O), δ(NH_2), 1206 – 1135 M v(CN); 877 w; 760 w; 684 w.

General method for the synthesis of the ^{99m}Tc(I) complexes *fac*-[^{99m}Tc(CO)$_3$(k^3-L)] (L = L^1 and L^2)

In a nitrogen-purged glass vial, 100 mL of a 10^{-4} M aqueous solution of the compounds **L1** or **L2** were added to 900 mL of a solution of the organometallic precursor fac-[^{99m}Tc(CO)$_3$(H$_2$O)$_3$]$^+$ (1 – 2 mCi) in saline or phosphate buffer pH 7.4. The reaction mixture was then heated to 100 ºC for 30min, cooled on an ice bath and the final solution analyzed by RP-HPLC. Retention times: 25.9 min (**Tc1**), 26.6 min (**Tc2**). Complexes **Tc3** - **Tc5** were prepared and characterized as described previously.[22, 28]

In vitro stability studies in plasma

100 mL of **Tc1** or **Tc2** were added to 500 µl of human plasma and incubated at 37 ºC. After 24 h, aliquots (100 µl) were taken and the plasmatic proteins precipitated with ethanol (200 µl). The

plasma was centrifuged at 3000 rpm for 15 min at 4ºC and the supernatant (protein-free plasma) filtered through a Millipore filter (0.22 µm), and analyzed by RP-HPLC.

Enzymatic assays

The iNOS activity assay was based on the method of hemoglobin assay previously described by Hevel and Marletta with slight modifications.[33, 34] The kinetics parameters for iNOS were determined using initial rate analysis. Initial rate data were fitted to irreversible single substrate Michaelis–Menten models. The kinetic parameters were determined using the direct linear plot of Eisenthal and Cornish-Bowden and the Hyper software (J.S. Easterby, University of Liverpool, UK; http://www.liv.ac.uk/~jse/software.html).[35] This method was chosen primarily because of its robustness.[36] The Km values represent a mean of triplicate measurements. Standard deviations of ± 5 to 10 % were observed.

Preparation of oxyhemoglobin

Oxyhemoglobin was prepared using a previously described protocol with some modifications.[37] Briefly, bovine hemoglobin in 50 mM HEPES pH 7.4 was reduced to oxyhemoglobin with 10-fold molar excess of sodium dithionite. The sodium dithionite was later removed by dialysis against 50 volumes of HEPES buffer for 18 h at 4 °C. The buffer was replaced 3 times. The concentration of oxyhemoglobin was determined spectrophotometrically using $\varepsilon_{415\ nm}$ = 131 mM^{-1} cm^{-1}. Oxyhemoglobin was stored at - 80 °C before use.

Determination of K_m values

All initial velocity measurements were recorded at 37 °C. Total reaction volumes were 1500 mL and contained 50 mM HEPES pH 7.4, 6 mM oxyhemoglobin, 200 mM NADPH, 10 mM Tetrahydrobiopterin (BH_4), 100 mM DTT and increasing concentrations of L-Arg, **L1**, **L2**, **Re1** and **Re2** (20 – 500 mM). Magnetic stirring in the spectrophotometer cuvette was essential to maintain isotropic conditions.

Reactions were initiated by the addition of iNOS enzyme (~1 U) to the pre warmed cuvette (~ 5 min). The NO-mediated conversion of oxyhemoglobin to methemoglobin was followed by monitoring the increase in absorbance at dual wavelength (401 and 421 nm) for 10 min.[38] Controls were performed in the same conditions without iNOS enzyme.

Cell culture

RAW 264.7 macrophages, and the following human tumoural cell lines: HeLa cervical cancer, A375 melanoma, MDA-MB-231 breast cancer and PC3 prostate cancer were grown in Dulbecco's Modified Eagle Medium (DMEM) with GlutaMax I supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1 % penicillin/streptomycin antibiotic solution (all from Invitrogen, UK). Cells were cultured in a humidified atmosphere of 95 % air and 5 % CO_2 at 37 °C, with the medium changed every other day.

Cellular uptake and internalization

Cellular uptake assays with **Tc1 - Tc5** were performed in HeLa, A375, MDA-MB-231 and PC3 cell lines seeded at a density of 0.2 million/well in a 24-well tissue culture plates. Cells were allowed to attach overnight. On the following day cells were exposed to complexes (about 200000 cpm in 0.5 mL of assay medium: Modified Eagle's Medium with 25 mM HEPES and 0.2% BSA) for a period of 5 min to 4 h. Incubation was terminated by removing the Tc complexes and by washing cells twice with ice-cold PBS with 0.2% BSA. Then, cells were lysed by 10 min incubation with 1 M NaOH at 37°C and the activity of lysates measured were in a γ-counter. The percentage of cell-associated radioactivity was calculated and represented as a function of incubation time. Uptake studies were carried out using at least four replicates for each time point.

The cellular uptake of **Tc2** was also evaluated in the presence of N-ethylmaleimide (NEM), a specific inhibitor of system y+. Cells were incubated with solutions of **Tc2** containing NEM (5 mM) during different periods (5, 15, 30 and 60 min) at 37ºC. The general procedure of cellular uptake was

followed. The inhibition of cellular uptake was expressed in percentage of the uptake of **Tc2** in the absence of inhibitor.

Internalization assays of **Tc2** complex were performed in HeLa, A375 and MDA-MB-231 cell lines seeded at a density of 0.2 million/well in a 24-well tissue culture plates. On the following day, cells were exposed to **Tc2** (about 200000 cpm in 0.5 mL of assay medium) for a period of 5 min to 4h. Incubation was terminated by washing the cells with ice-cold assay medium. Cell surface-bound **Tc2** was removed by two steps of acid wash (50 mM glycine·HCl/100 mM NaCl, pH 2.8) at room temperature for 4 min. The pH was neutralized with cold PBS with 0.2% BSA. The cells were then lysed by 10 min incubation with 0.5 N NaOH at 37 °C to determine internalized **Tc2**. The activity of the lysates and cell surface-bound fractions were counted in a γ-counter.

Internalization assays for **Tc2** were also performed in the presence of L-Lysine. For this study, A375, HeLa and MDA-231 cells were incubated 2h at 37ºC with solutions of **Tc2** containing different concentrations of L-Lysine (0, 0.5, 1, 5 and 10mM).

Cell viability determination

Cell viability was evaluated by using a colorimetric method based on the tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), which is reduced by viable cells to yield purple formazan crystals.[39] RAW 264.7 macrophages in DMEM medium without arginine supplemented with 10% FBS were seeded in 96-well plates at a density of 9 x 10^4 cells per well, immediately induced with LPS (2 mg/mL) for 4 h, and then incubated for 24 h in the presence of the compounds (500 μM) and arginine (when indicated).[22] At the end of the incubation period the media was removed and the cells were incubated with MTT (0.5 mg/mL in culture medium; 200 mL) for 3 - 4 h at 37 °C and 5 % CO_2. The purple formazan crystals formed inside the cells were then dissolved in 200 mL of DMSO by thorough shaking, and the absorbance was read at 570 nm, using a plate spectrophotometer (Power Wave Xs; Bio-Tek). Each test was performed with at least six replicates

and repeated at least 2 times. The result was expressed as percentage of the surviving cells in relation with the control.

Assay of iNOS activity in vivo

RAW 264.7 macrophages in DMEM medium without arginine supplemented with 10% FBS DMEM, were plated at a density of 9 x 10^4 cells per well in 96-well plates. Cells were immediately induced with 10 μL of LPS (2 μg/mL in PBS) for 4 h, and then incubated for 24 h in the presence of the compounds (500 μM). At the end of the incubation period, the culture medium was collected and assayed for nitrite production using the commercially available Griess reagent (1 % sulfanilamide, 0.1 % *N*-1-naphthyl ethylenediamine, 2.5 % orthophosphoric acid; Sigma-Aldrich). Briefly, 50 μL of Griess reagent was mixed with an equal volume of medium at room temperature and absorbance was measured at 540 nm after 10 min. Fresh culture medium served as the blank in all experiments. Each experiment was performed with six replicates and repeated three times.

Western blot

Western blot experiments were performed to demonstrate the expression of CAT-1 in the four human tumoural cell lines (A375, HeLa, MDA-MB-231 and PC3). Cells were lysed with CelLytic™ M Extraction Reagent (Sigma) supplemented with Complete Protease Inhibitor Cocktail tablets (Roche). After 15 min at 4° C with regular shaking, lysates were collected and centrifuged at 14000 g for 10 min to pellet the cellular debris, and the supernatants removed for further use. The total protein content was determined using the DC Protein Assay Kit (BioRad). Samples (75 μg of protein) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. Blots were blocked with 5% nonfat dry milk in PBS-T for 2 h and then incubated overnight with the primary antibodies against CAT-1 (1:200, Santa Cruz, sc-66824) and actin (1:15000, Sigma, A3853). Membranes were washed with PBS-T and incubated for 1 h with the secondary antibodies in a 1:3000 dilution (goat anti-rabbit IgG-HRP for α-CAT-1 and goat

anti-mouse IgG-HRP for α-actin, BioRad). Membranes were developed using the SuperSignal West Pico Substrate kit (Pierce, Rockford, IL) according to the manufacturer's instructions.

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References

1. S. L. Pimlott and A. Sutherland, *Chemical Society Reviews*, 2011, **40**, 149-162.
2. S. N. Histed, M. L. Lindenberg, E. Mena, B. Turkbey, P. L. Choyke and K. A. Kurdziel, *Nuclear Medicine Communications*, 2012, **33**, 349-361.
3. H. Schoder and S. M. Larson, *Seminars in Nuclear Medicine*, 2004, **34**, 274-292.
4. A. Sundin, U. Garske and H. Orlefors, *Best Practice & Research Clinical Endocrinology & Metabolism*, 2007, **21**, 69-85.
5. J. McConathy and M. M. Goodman, *Cancer and Metastasis Reviews*, 2008, **27**, 555-573.
6. J. McConathy, W. Yu, N. Jarkas, W. Seo, D. M. Schuster and M. M. Goodman, *Medicinal Research Reviews*, 2012, **32**, 868-905.
7. R. Hyde, P. M. Taylor and H. S. Hundal, *Biochemical Journal*, 2003, **373**, 1-18.
8. E. I. Closs, J. P. Boissel, A. Habermeier and A. Rotmann, *Journal of Membrane Biology*, 2006, **213**, 67-77.
9. C. Huang and J. McConathy, *Journal of Nuclear Medicine*, 2013, **54**, 1007-1010.
10. K. K. S. Sai, C. Huang, L. Yuan, D. Zhou, D. Piwnica-Worms, J. R. Garbow, J. A. Engelbach, R. Mach, K. M. Rich and J. McConathy, *Journal of Nuclear Medicine*, 2013, **54**, 1120-1126.
11. E. I. Closs, A. Simon, N. Vekony and A. Rotmann, *Journal of Nutrition*, 2004, **134**, 2752S-2759S.
12. S. Moncada, R. M. J. Palmer and E. A. Higgs, *Pharmacological Reviews*, 1991, **43**, 109-142.
13. D. Fukumura, S. Kashiwagi and R. K. Jain, *Nature Reviews Cancer*, 2006, **6**, 521-534.
14. P. Mukherjee, M. A. Cinelli, S. Kang and R. B. Silverman, *Chemical Society Reviews*, 2014, **43**, 6814-6838.
15. M. M. Phillips, M. T. Sheaff and P. W. Szlosarek, *Cancer Research and Treatment*, 2013, **45**, 251-262.
16. N. Gupta, S. Miyauchi, R. G. Martindale, A. V. Herdman, R. Podolsky, K. Miyake, S. Mager, P. D. Prasad, M. E. Ganapathy and V. Ganapathy, *Biochimica Et Biophysica Acta-Molecular Basis of Disease*, 2005, **1741**, 215-223.
17. N. Gupta, P. D. Prasad, S. Ghamande, P. Moore-Martin, A. V. Herdman, R. G. Martindale, R. Podolsky, S. Mager, M. E. Ganapathy and V. Ganapathy, *Gynecologic Oncology*, 2006, **100**, 8-13.

18. S. Karunakaran, S. Ramachandran, V. Coothankandaswamy, S. Elangovan, E. Babu, S. Periyasamy-Thandavan, A. Gurav, J. P. Gnanaprakasam, N. Singh, P. V. Schoenlein, P. D. Prasad, M. Thangaraju and V. Ganapathy, *Journal of Biological Chemistry*, 2011, **286**, 31830-31838.
19. Y. Lu, W. Wang, J. Wang, C. Yang, H. Mao, X. Fu, Y. Wu, J. Cai, J. Han, Z. Xu, Z. Zhuang, Z. Liu, H. Hu and B. Chen, *Plos One*, 2013, **8**.
20. S. A. Abdelmagid, J. A. Rickard, W. J. McDonald, L. N. Thomas and C. K. L. Too, *Journal of Cellular Biochemistry*, 2011, **112**, 1084-1092.
21. Y. Liu, J. K. Pak, P. Schmutz, M. Bauwens, J. Mertens, H. Knight and R. Alberto, *Journal of the American Chemical Society*, 2006, **128**, 15996-15997.
22. B. L. Oliveira, P. D. Raposinho, F. Mendes, F. Figueira, I. Santos, A. Ferreira, C. Cordeiro, A. P. Freire and J. D. G. Correia, *Bioconjugate Chemistry*, 2010, **21**, 2168-2172.
23. A. de Almeida, B. L. Oliveira, J. D. G. Correia, G. Soveral and A. Casini, *Coordination Chemistry Reviews*, 2013, **257**, 2689-2704.
24. B. L. Oliveira, I. S. Moreira, P. A. Fernandes, M. J. Ramos, I. Santos and J. D. G. Correia, *Journal of Molecular Modeling*, 2013, **19**, 1537-1551.
25. B. L. Oliveira, I. S. Moreira, P. A. Fernandes, M. J. Ramos, I. Santos and J. o. D. G. Correia, *Journal of Molecular Graphics and Modelling*, 2013, **45**, 13-25.
26. B. L. Oliveira, M. Morais, F. Mendes, I. S. Moreira, C. Cordeiro, P. A. Fernandes, M. J. Ramos, R. Alberto, I. Santos and J. D. G. Correia, *Chemical Biology & Drug Design*, 2015, **86**, 1072-1086.
27. M. Morais, A. Paulo, L. Gano, I. Santos and J. D. G. Correia, *Journal of Organometallic Chemistry*, 2013, **744**, 125-139.
28. B. L. Oliveira, J. D. G. Correia, P. D. Raposinho, I. Santos, A. Ferreira, C. Cordeiro and A. P. Freire, *Dalton Transactions*, 2009, 152-162.
29. B. L. Oliveira, J. D. G. Correia, P. D. Raposinho, I. Santos, A. Ferreira, C. Cordeiro and A. P. Freire, *Dalton Transactions*, 2009, **0**, 152-162.
30. M. Pan, H. A. Choudry, M. J. Epler, Q. H. Meng, A. Karinch, C. M. Lin and W. Souba, *Journal of Nutrition*, 2004, **134**, 2826S-2829S.
31. J. MacMicking, Q. W. Xie and C. Nathan, *Annual Review of Immunology*, 1997, **15**, 323-350.
32. N. Lazarova, S. James, J. Babich and J. Zubieta, *Inorganic Chemistry Communications*, 2004, **7**, 1023-1026.
33. J. Hevel and M. Marletta, *Methods Enzymol.*, 1994, **233**, 250 - 258.
34. S. Archer, *The FASEB Journal*, 1993, **7**, 349-360.
35. Robert Eisenthal and A. Cornish-Bowden, *Biochem J.*, 1974, **139**, 715 – 720.
36. A. Cornish-Bowden, *Fundamentals of Enzyme Kinetics*, Portland Press, London, 2nd edn., 1995.
37. D. J. Wolff, D. S. Gauld, M. J. Neulander and G. Southan, *Journal of Pharmacology and Experimental Therapeutics*, 1997, **283**, 265-273.
38. R. G. Knowles and S. Moncada, *Biochemical Journal*, 1994, **298**, 249-258.
39. T. Mosmann, *Journal of Immunological Methods*, 1983, **65**, 55-63.