ECB 2021
The 1st International Electronic Conference on Biomedicine
01–26 JUNE 2021 | ONLINE

Protein nanocages for anticancer metal-based drug delivery

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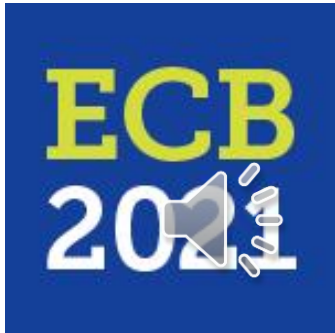
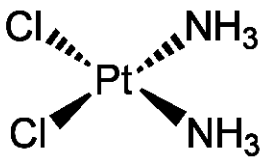
FONDAZIONE AIRC PER LA RICERCA SUL CANCRO

Abstract: Supramolecular protein assembly can be used as reaction vessels for drug delivery. Ferritin (Ft) is a ferroxidase that forms a spherical nanocage involved in cellular iron storage and detoxification. It is very promising as a drug loading and releasing system since it is bio-compatible and highly stable. It can be internalized via Ft-binding receptors over-expressed on different cancer cells. Well established metallodrugs have been trapped within the Ft nanocages, taking advantage of the alkaline pH disassembly/reassembly protocol. The drug-loaded nanocomposites have been characterized to evaluate the protein secondary structure content upon drug encapsulation, and to assess the drug loading within the protein cage. The amount of drug trapped inside the nanocage has been quantified, and metallodrugs binding sites and the nature of their interaction with Ft have been unveiled. The compounds often degrade upon encapsulation and metal-containing fragments coordinate Cys or His residues. However, many metallodrugs molecules remain trapped in the bulk. Biological activity studies show that the presence of the cage reduces the overall toxicity of the metallodrugs, but increases their selectivity towards cancer cells. Altogether these data indicate that encapsulation of metal-based drugs within Ft nanocages is a promising strategy to deliver these molecules to their final targets.

Keywords: protein cages; drug delivery; metallodrugs; ferritin.



Cisplatin



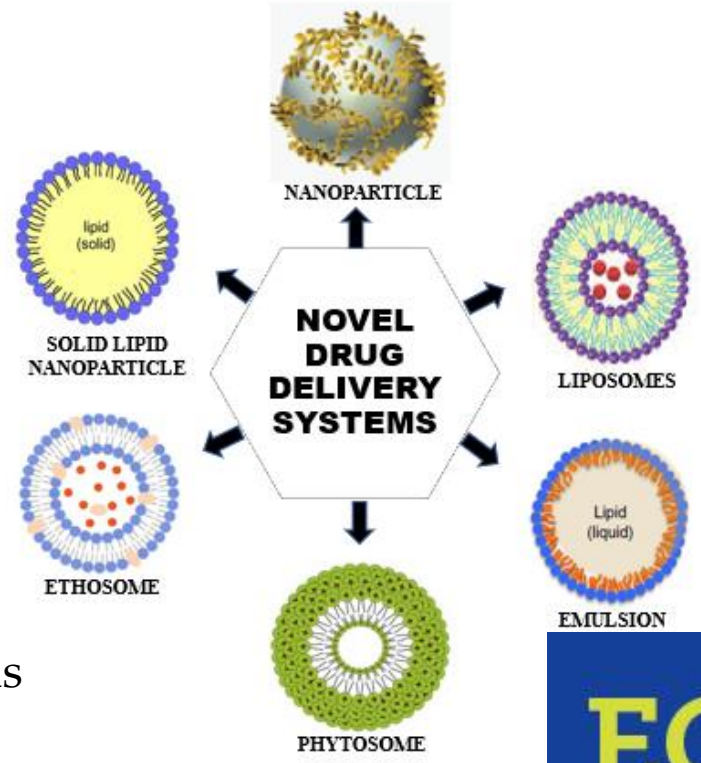
Rosenberg B. Nature (1965) 205, 698

Metal compounds have an important place in the clinical practice

They are used as drugs to treat several human diseases (carcinomas, lymphomas, infection control, anti-inflammatory, diabetes, and neurological disorders).

Several side effects: general toxicity and drug resistance

- ✓ alternative metal centers
- ✓ change of oxidation state and coordination geometry
- ✓ ligand modification
- ✓ development of polynuclear systems
- ✓ *drug delivery/targeting protocols*



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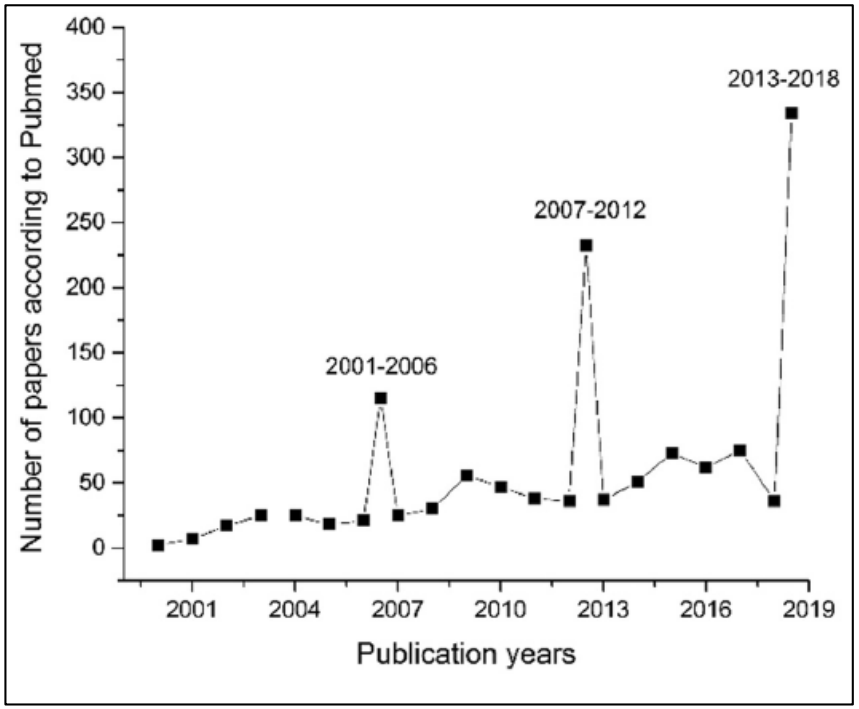
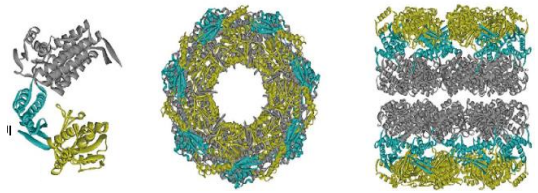
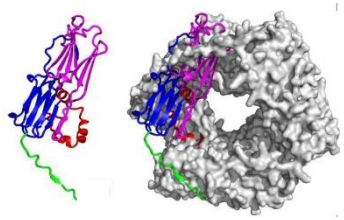
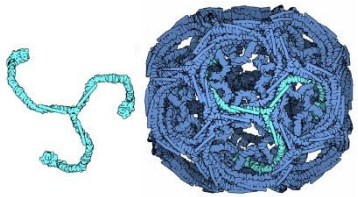
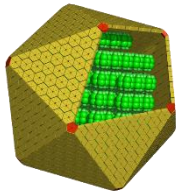
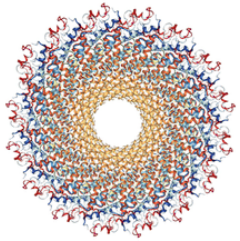
Proteins as delivery systems for anticancer drugs



A graph on the growing interest of researchers in the field of "proteins as delivery systems for anticancer drugs" from 2000 to 2018 (data from Pubmed).

* chaperonines 
* heat shock proteins 
* clathrin 
* carboxysomes 
* mosaic viruses 

Monti, D.M. *et al*. Nanomedicine: NBM (2019) 20, 101997

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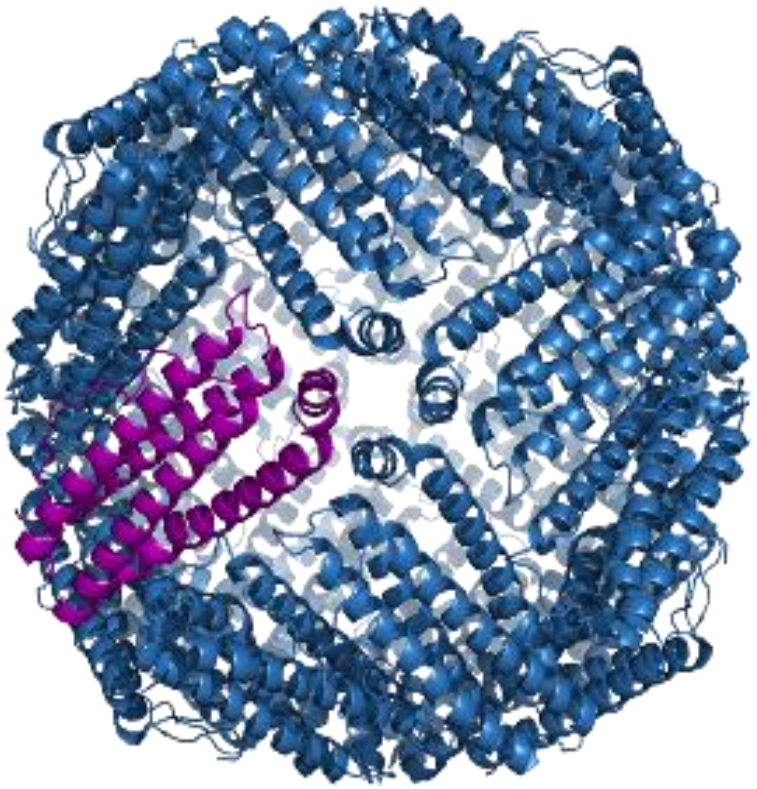
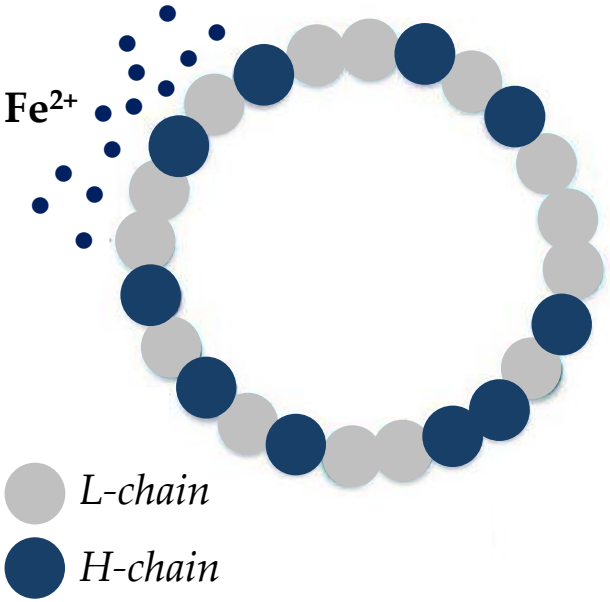
Use of a delivery system/carrier for the drug

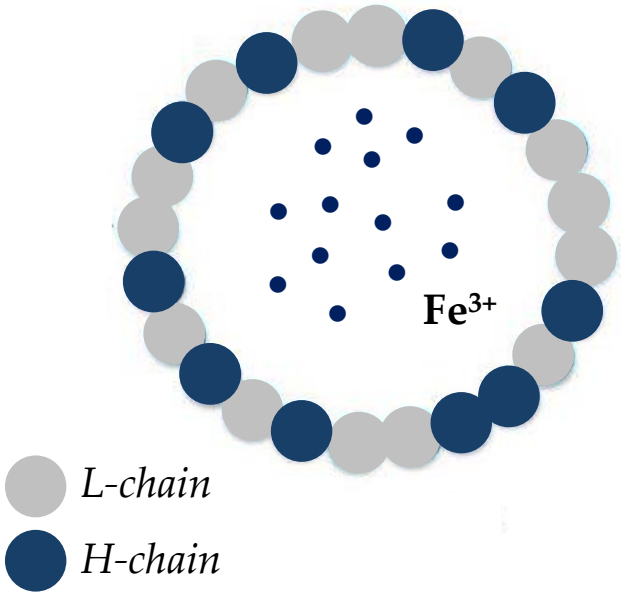


horse spleen ferritin

Apo-Ferritin



Holo-Ferritin



- 24-mer cage with octahedral symmetry
- outer diameter ~120 Å - inner diameter ~80 Å
- 2 type of chains: H-chain (heavy) + L-chain (light)
- four-helix bundle
- iron sequestration → detoxification and cellular reserve

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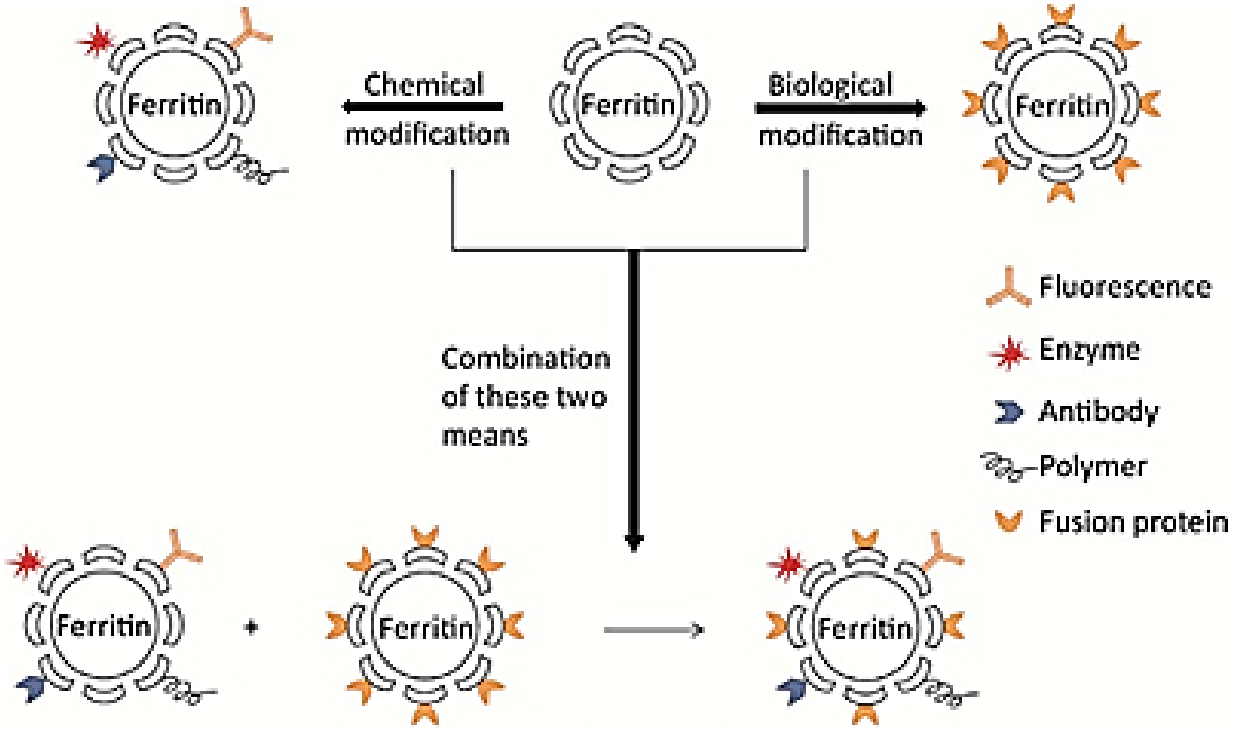
- biocompatible and non-immunogenic
- stable and soluble in the bloodstream

Why ferritin?

- **amenable to both genetic and chemical functionalization**



- could lead to longer circulation half-life and to better tumor accumulation rates

Truffi, M. et al. Pharmacological Research (2016) 170: 57

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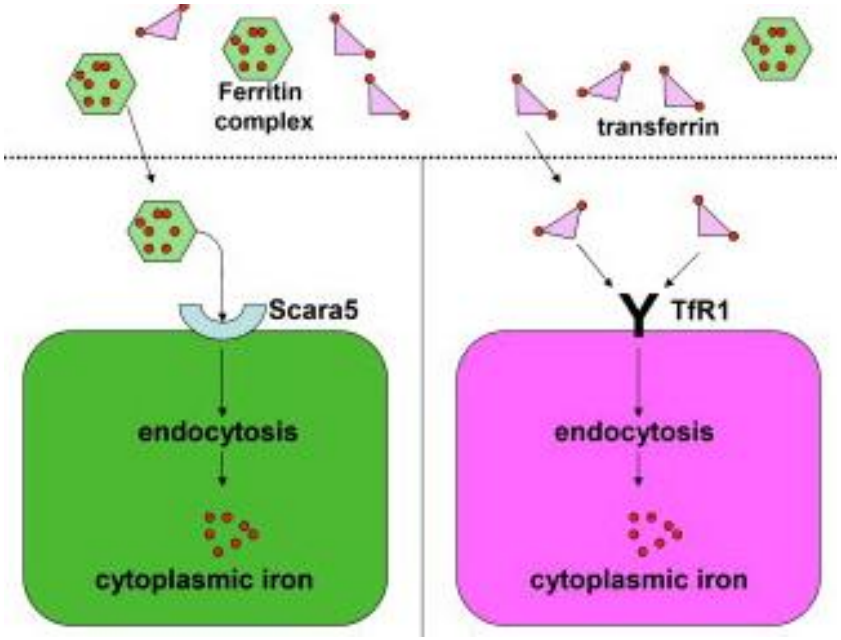
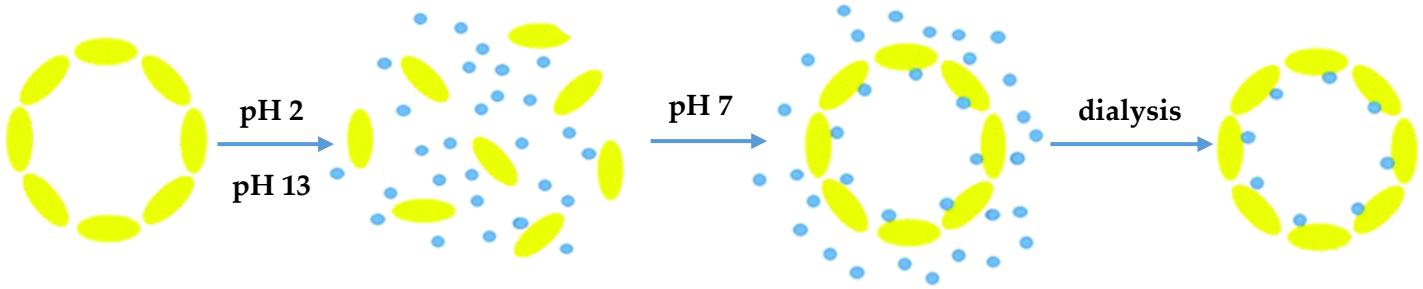
- recognized by receptors over-expressed on cancer cells surface



- easy assembly/disassembly by pH modulation



Uchida, M. et al. Biochimica et Biophysica acta (2010) 1800: 834; Li et al. PNAS (2010) 107: 3505-3510.

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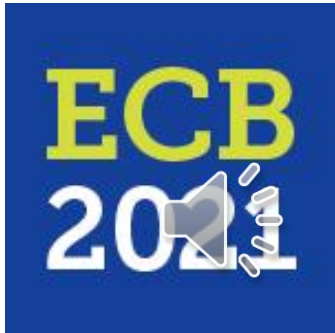
To encapsulate selected metal-based compounds in a protein-based drug delivery system

- To investigate in details the interactions between selected metal-based compounds and the protein

- To find out a way to improve the pharmacological profile of the investigated metallodrugs, trying to reduce their general toxicity and to increase target specificity



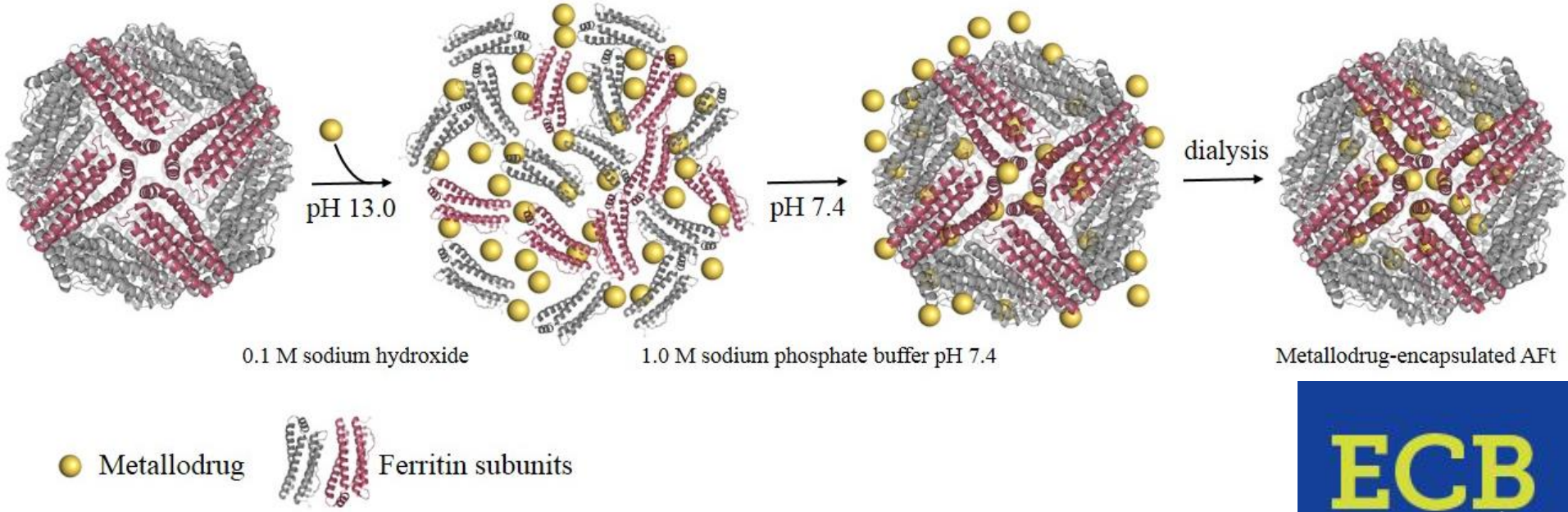
Alkaline pH encapsulation protocol

The ferritin cage is disassembled at alkaline pH in the presence of 0.1 M NaOH

The metallodrug is added to the ferritin solution and the reaction mixture is incubated for 1h under stirring

After the incubation, the pH is restored to 7.4 by adding 1.0 M sodium phosphate buffer

The metallodrug-loaded ferritin is dialyzed and then stored at 4°C



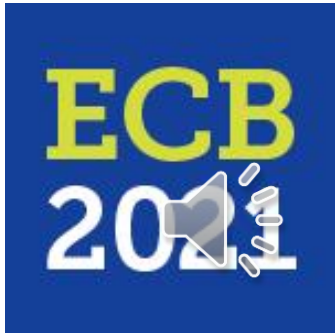
Pontillo, N. *et al.* **Chem Commun** (2016) 52, 4136

DRUG-LOADED NANOCARRIERS CHARACTERIZATION

- *From a structural and biophysical point of view*, analysing the conformation of the nanocomposites and solving their X-ray structures

- *From an analytical point of view*, defining the exact amount of metal encapsulated inside the nanocage and the protein:metallodrug stoichiometry

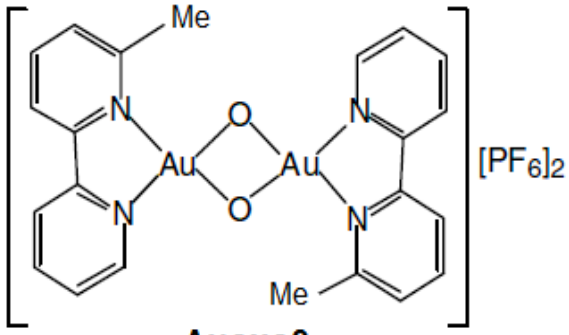
- *From a biological point of view*, evaluating the cytotoxicity on cancer as well as on non-cancer cells, studying the mechanism of the cellular uptake and the cellular pathways activated



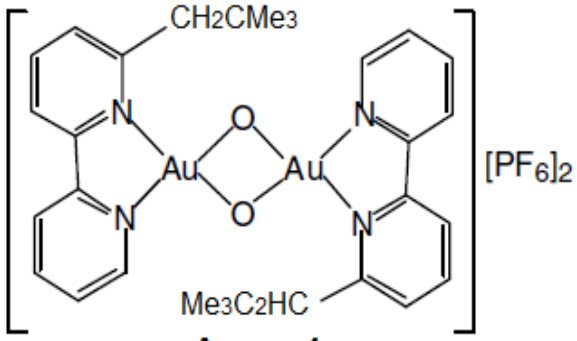
79 $4f^{14}5d^{10}6s^1$
Au
Gold
196.967

GOLD-based compounds

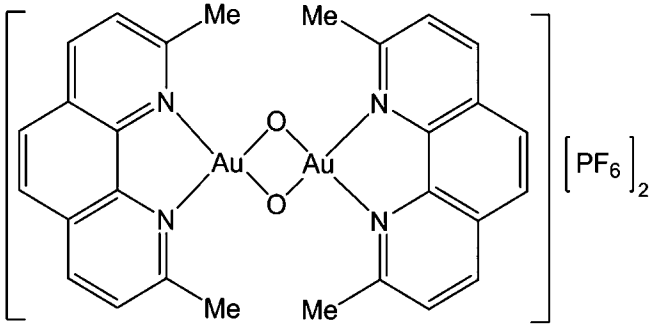
- Binuclear gold(III) oxo-bridged compounds supported by substituted 2,2′-bipyridine
  - Appreciable stability under physiological-like conditions
  - Antiproliferative effects toward selected human cancer cell lines



Auoxo3



Auoxo4

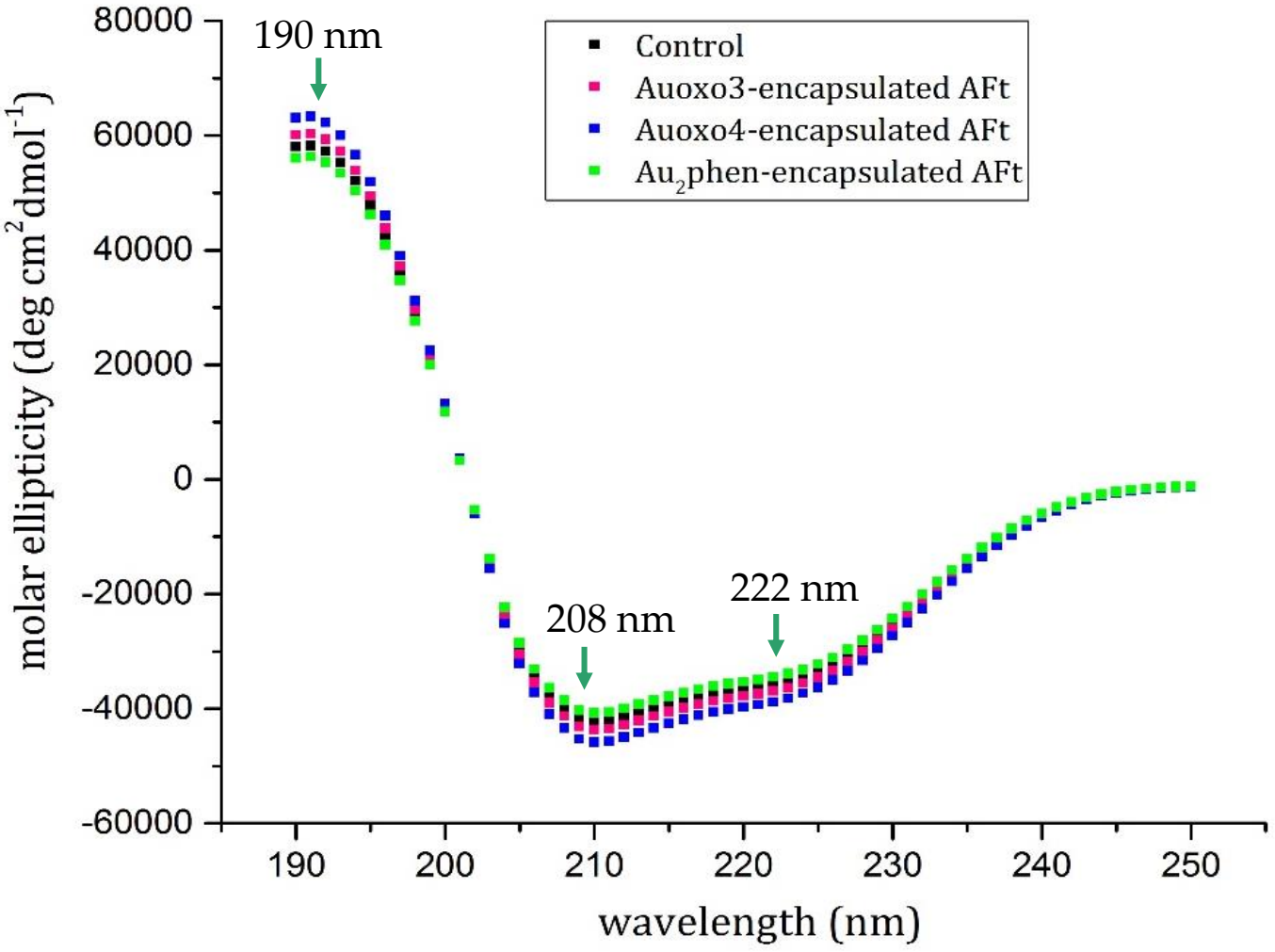


Au2phen

Gabbiani, C. *et al.* Gold Bullletin (2007) 40:1.

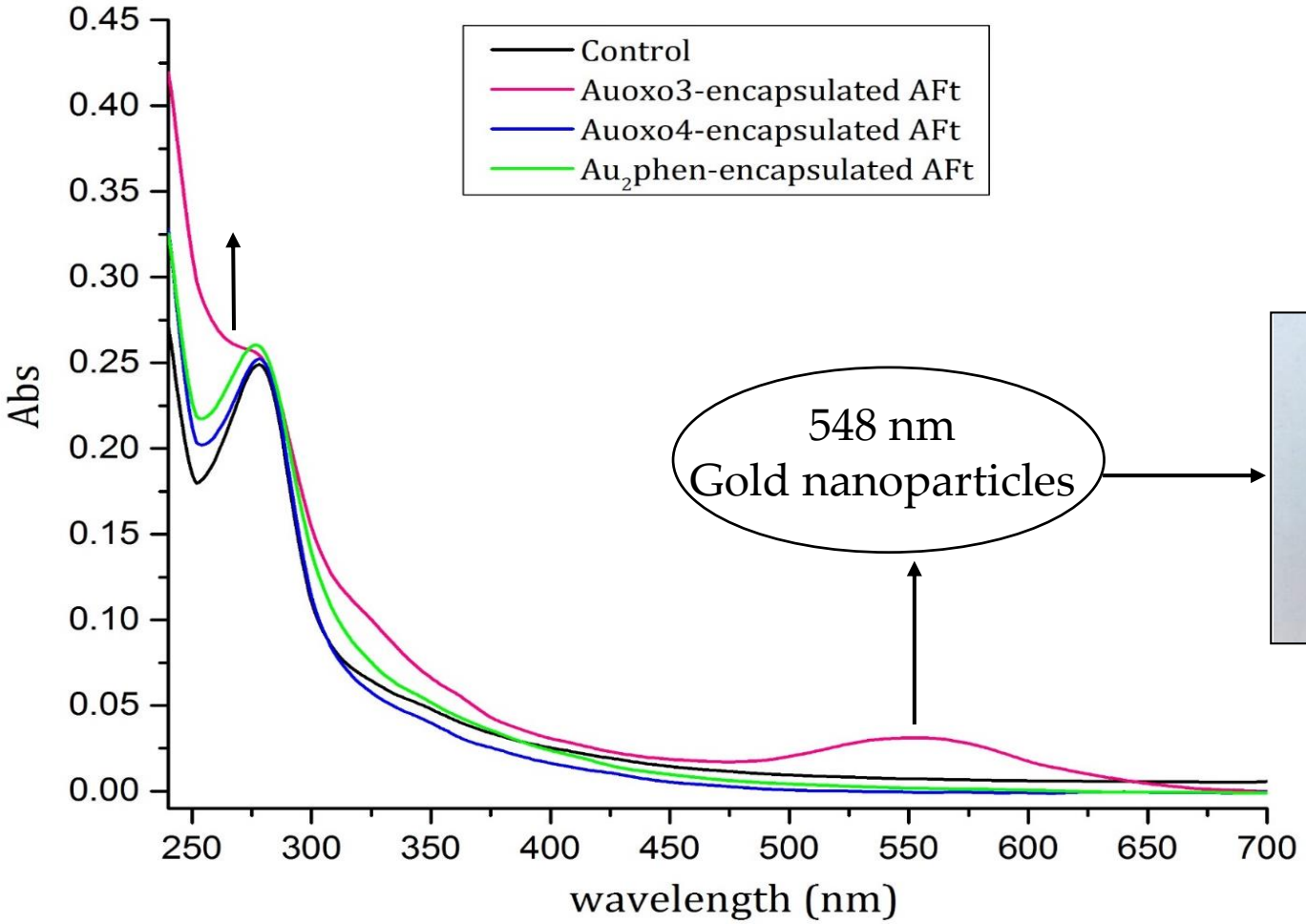
ECB 2011

Far UV-CD spectroscopy



Ferraro G. *et al.* **Chem Commun** Camb (2016) 52, 9518; Monti, D.M. *et al.* **Dalton Trans** (2017) 46, 15354.

UV-Vis absorption spectroscopy



548 nm
Gold nanoparticles

Ferraro G. *et al.* **Chem Commun** Camb (2016) 52, 9518; Monti, D.M. *et al.* **Dalton Trans** (2017) 46, 15354.

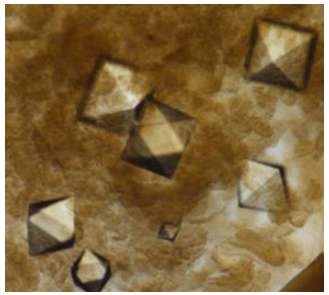
ICP- Mass Spectrometry measurements

SAMPLE	Au Atoms/Cage	Au Atoms/Single chain
Auoxo3-encapsulated-AFt	300 – 500	12.5 – 20.8
Auoxo4-encapsulated-AFt	384 – 432	16 – 18
Au2phen-encapsulated-AFt	384 – 432	16 – 18

Gold(III)-AFt adducts crystallization

Auoxo3-AFt

Auoxo4-AFt



Au2phen-AFt

Crystallization condition
0.1 – 0.2 ammonium sulphate
0.1 M Tris-HCl buffer pH 7.0 – 7.7
0.04 – 0.06 cadmium sulphate

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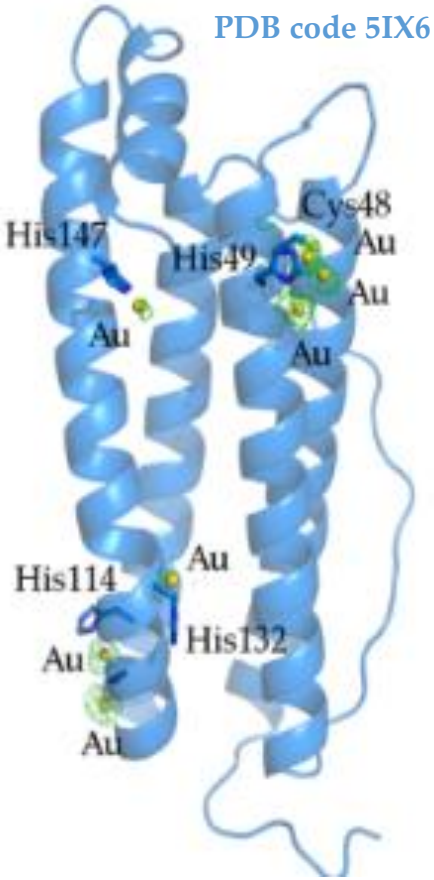
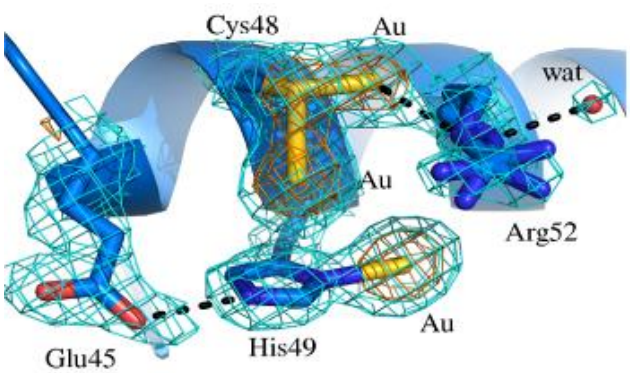
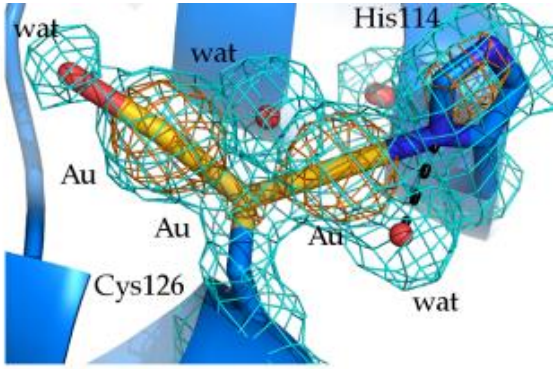
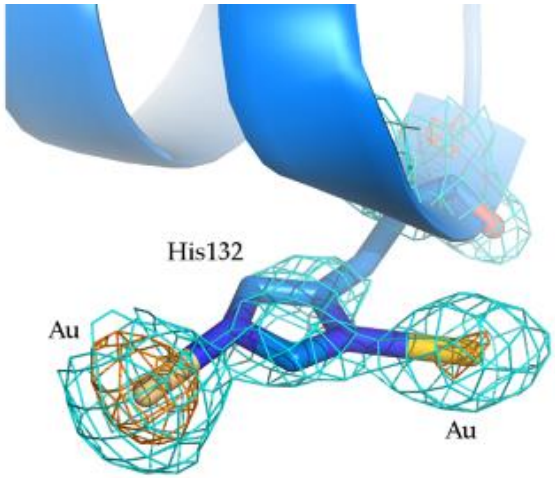
X-ray structure solution and refinement

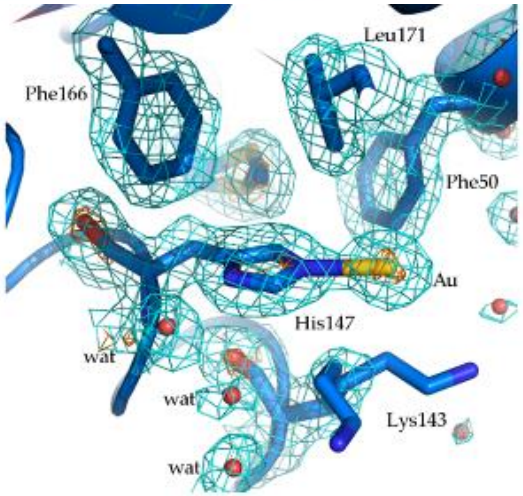
PDB code 5IX6











Auoxo3-encapsulated AFt → naked **Au(I) ions** bind to the side chains of:

- Cys48
- His49
- His114
- His114 and Cys126
- Cys126
- His132
- His147

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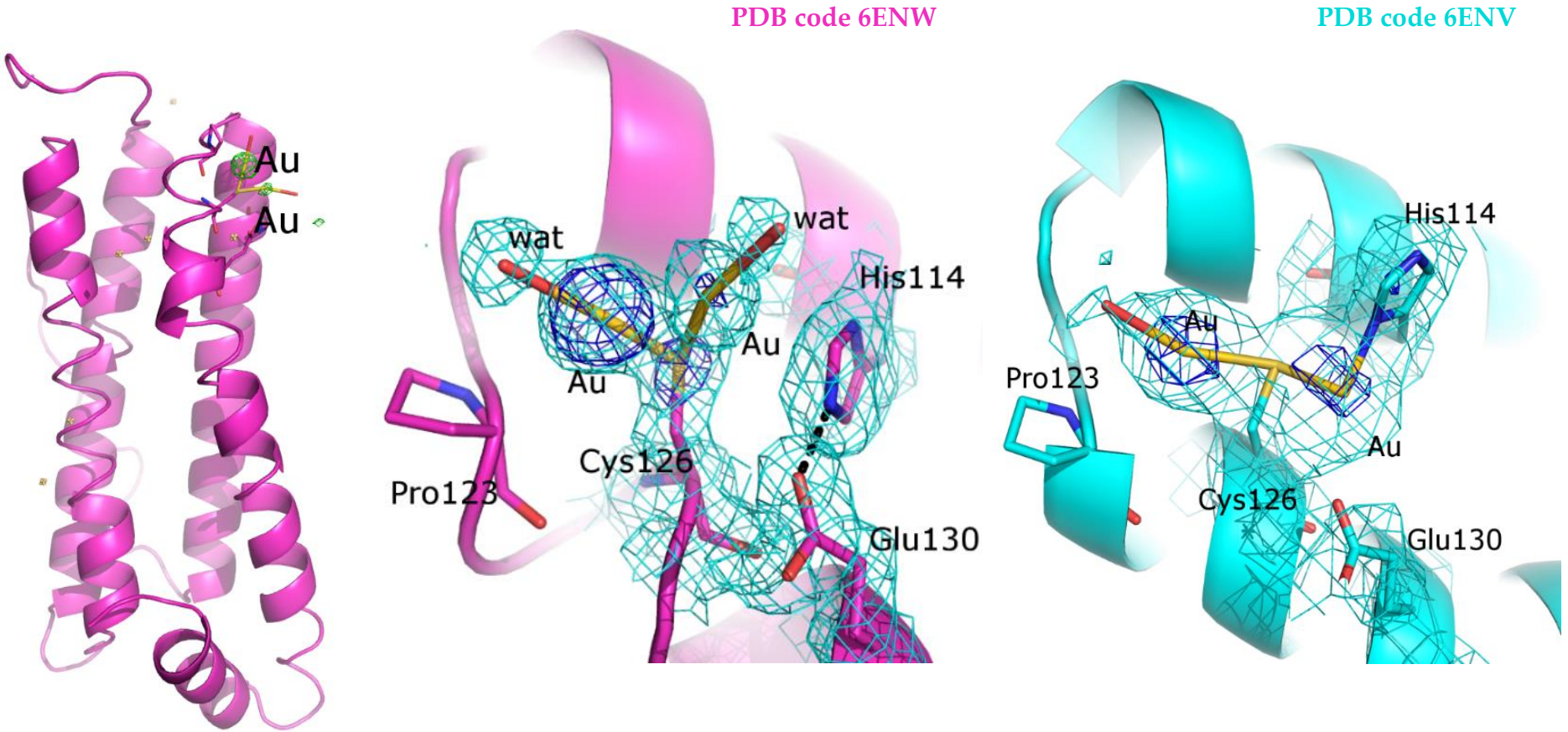
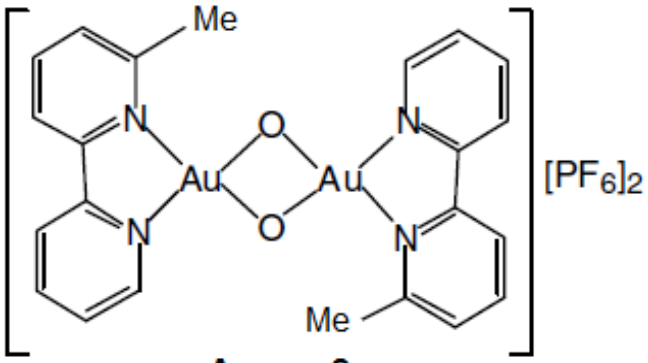
X-ray structure solution and refinement



Auoxo4-encapsulated AFt and **Au_2phen-encapsulated AFt**

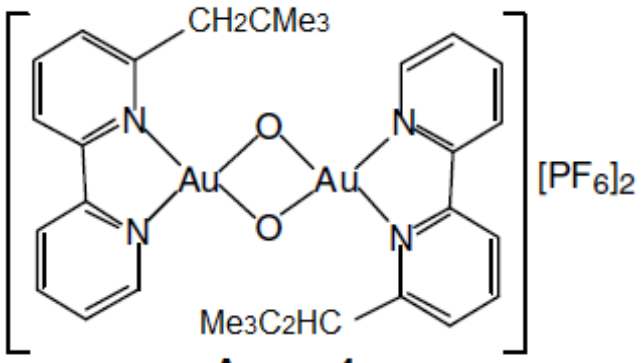
→ two **Au(I)** ions bound to side chains of Cys126.

Biophysical properties



Auoxo3

E_p = - 0.40 V



Auoxo4

E_p = - 0.39 V

RATE OF HYDROLYSIS REACTION

- ***Auoxo4*** $t_{1/2}$ = about 9 h
- ***Auoxo3*** $t_{1/2}$ = about 1 h

Auoxo4 *neopentyl group* → better protection from nucleophilic attack than **Auoxo3** *methyl group*

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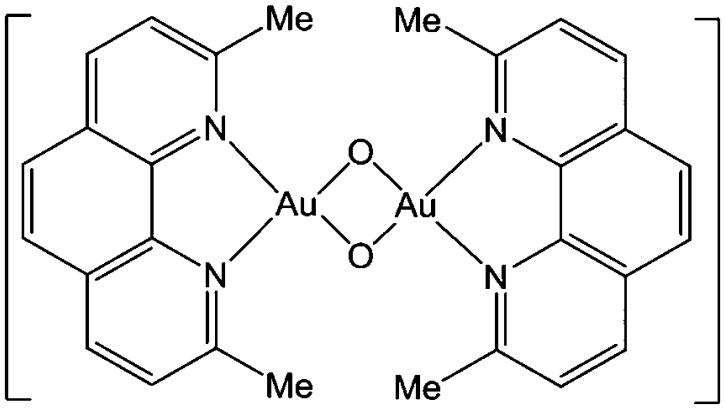
Biophysical properties



Au2phen

E_p = - 0.946 V

High redox stability

1. Lower number of gold binding sites in the structures of Auoxo4-AFt and Au2phen-AFt

2. Higher tendency of Auoxo3 to form gold nanoparticles

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Cytotoxic activity

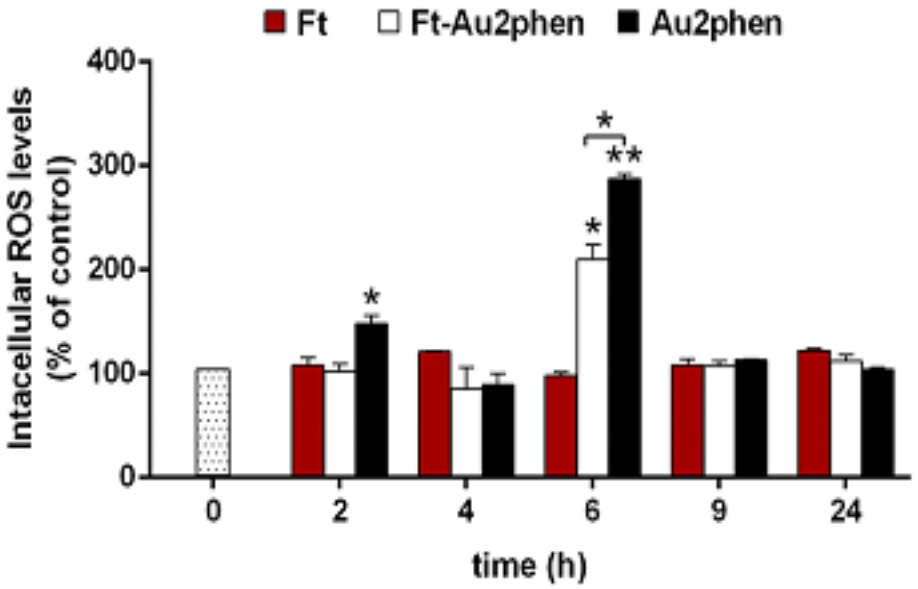
- **Cancer cell lines**
- **Normal cell lines**

After 72 h

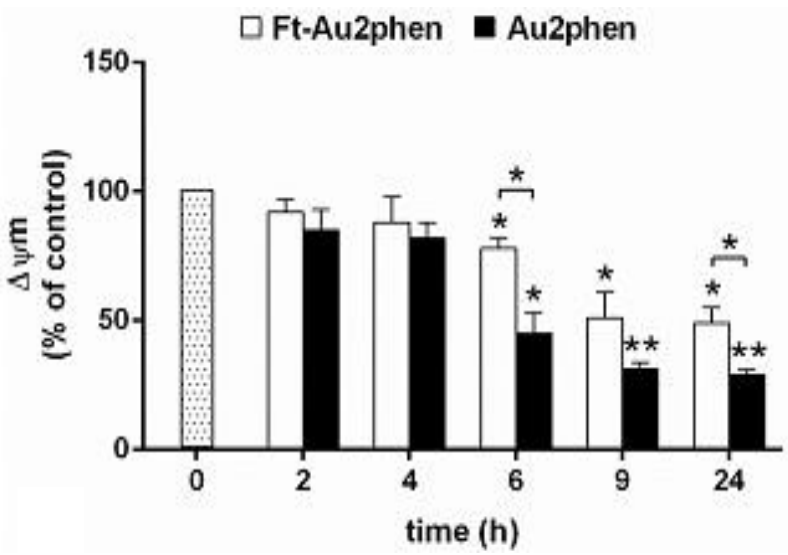
	AFt	Auoxo3	Auoxo3-encapsulated AFt
MCF-7 breast cancer cells	**>1000**	**8 ± 2**	**41 ± 9**
HeLa cervical cancer cells	**>1000**	**3 ± 1**	**42 ± 1**
H9c2 rat cardio-myoblast cells	>1000	4 ± 1	59 ± 10
HaCaT human keratinocyte cells	>1000	14.2 ± 0.7	69 ± 11

After 48 h

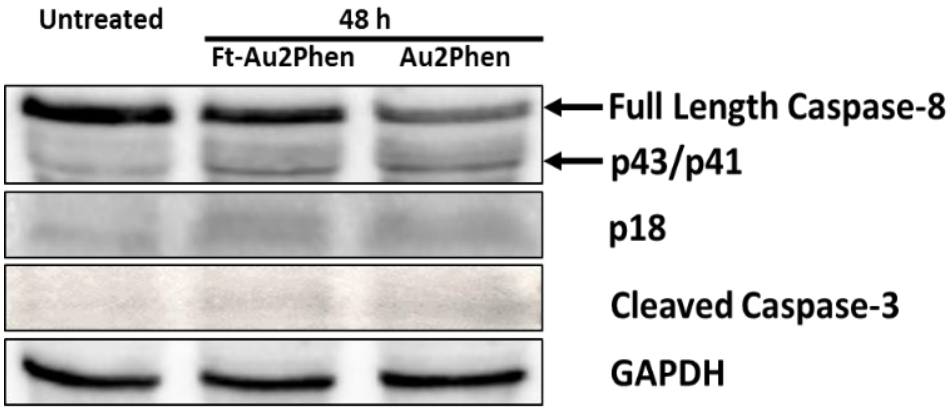
	Auoxo4	Auoxo4-encapsulated AFt	Au2phen	Au2phen-encapsulated AFt
MCF-7 breast cancer cells	**34.8 ± 4.3**	**30 ± 1**	**10.6 ± 2.5**	**6 ± 1**
HeLa cervical cancer cells	**13.7 ± 0.9**	**23 ± 9**	**6.5 ± 1.0**	**32 ± 4**
H9c2 rat cardio-myoblast cells	14.6 ± 1.4	68 ± 6	1.28 ± 0.06	40 ± 1
HaCaT human keratinocyte cells	12.4 ± 1.6	73 ± 6	14.3 ± 1.7	36 ± 9



ROS levels increase after 6 h of incubation with **Au_2phen-encapsulated AFt** and the drug alone



Dissipation of the mitochondrial membrane potential



Caspases 8 and 3 are activated after 48 h incubation → apoptosis is triggered by the release of the drug inside the cell

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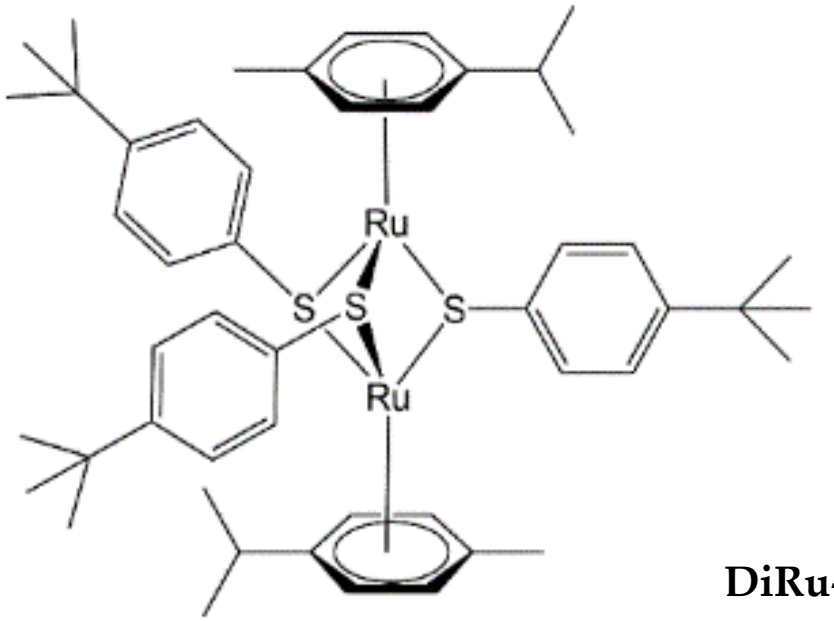
RUTHENIUM-based compound

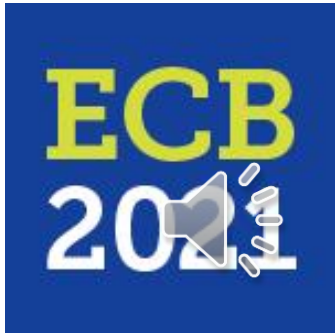


DiRu-1

- Ru-arene complex belonging to the family of dinuclear trithiolato complexes of the general type $[(arene)_2Ru_2(SR)_3]^+$

- Highly cytotoxic towards selected cancer cell lines

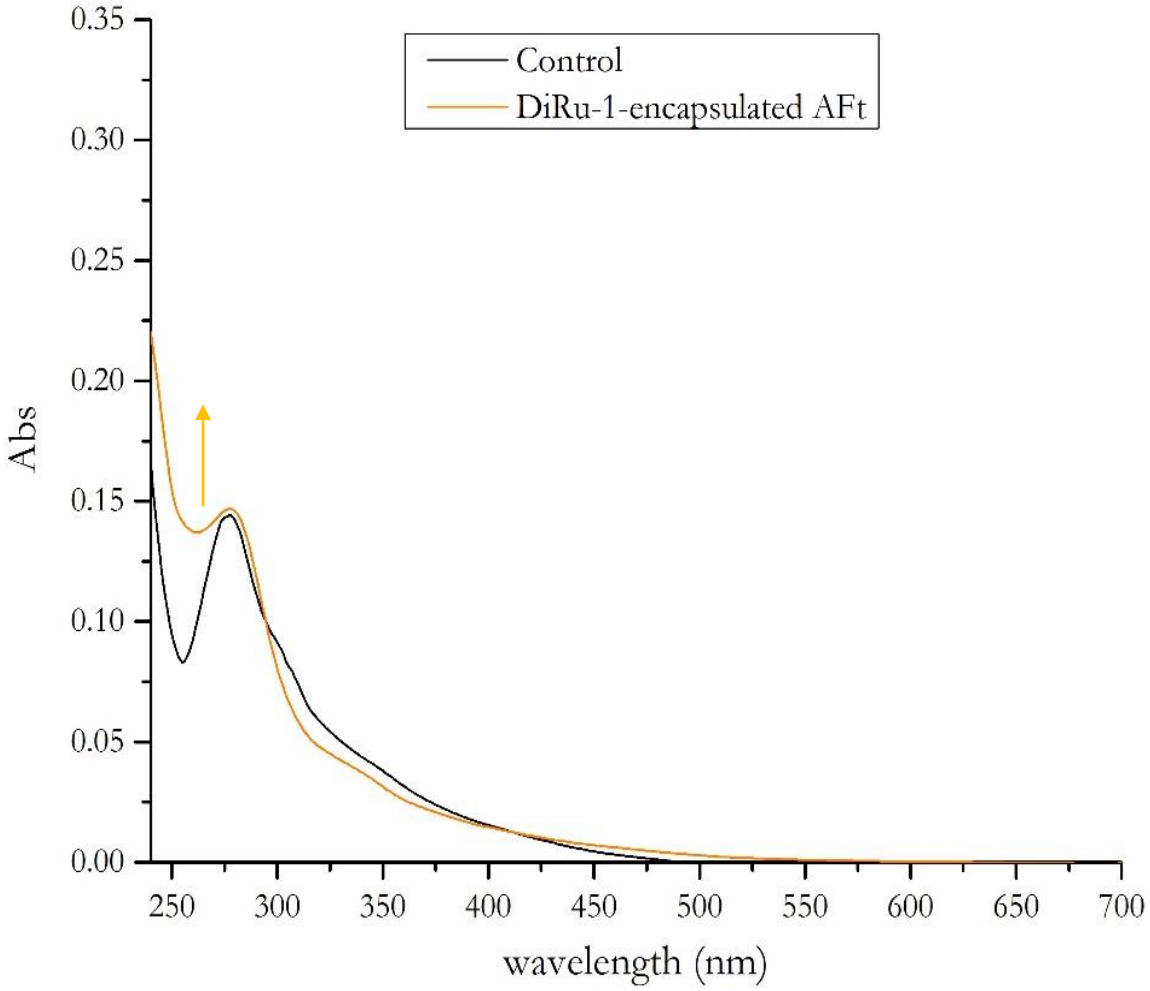
Furrer J. *et al.* **Coord Chem Rev** (2016) 309, 36.



ICP-MS measurements

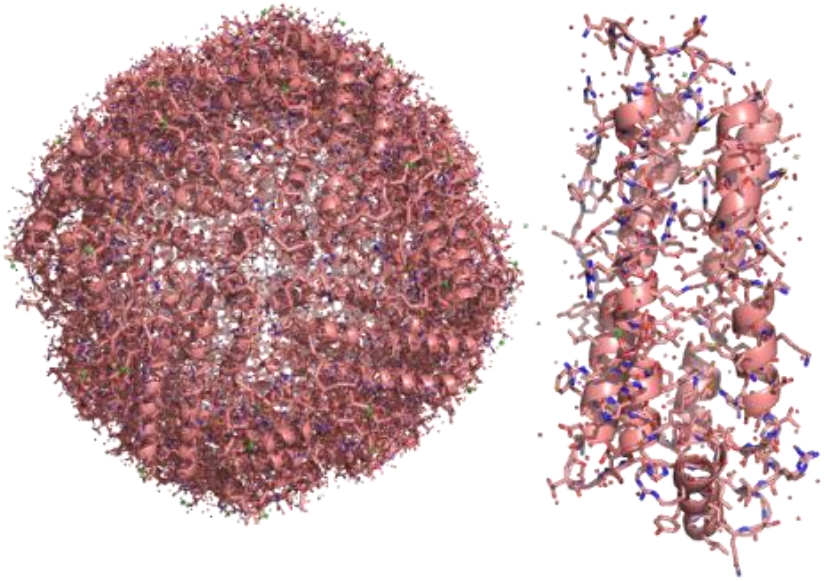
1:3 protein chain to Ru ratio (3.3 ± 0.7)

*~72 Ru atoms/cage → 36 molecules of **DiRu-1**/cage*



A broad intense absorption signal in the **250–300 nm** range, distinctly separated from the background signal of the control, is characteristic of the interactions between proteins and several metal-based drugs.

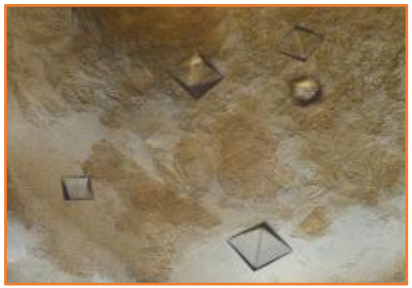
X-ray structure solution and refinement



*Ru atoms are not directly bound to any protein residue side chains in the structure of **DiRu-1-encapsulated AFt**.*

- **DiRu-1** is trapped within the cage
- Only weak interactions (long-range electrostatic interactions) exist between **DiRu-1** and AFt

(drug-free) AFt and **DiRu-1-encapsulated AFt CRYSTALS** washed with the reservoir and dissolved in 20 µL of milliQ water



ICP-MS measurements

- **DiRu-1**-encapsulated AFt crystals → 37 ng Ru
- AFt (drug-free) crystals → undetectable

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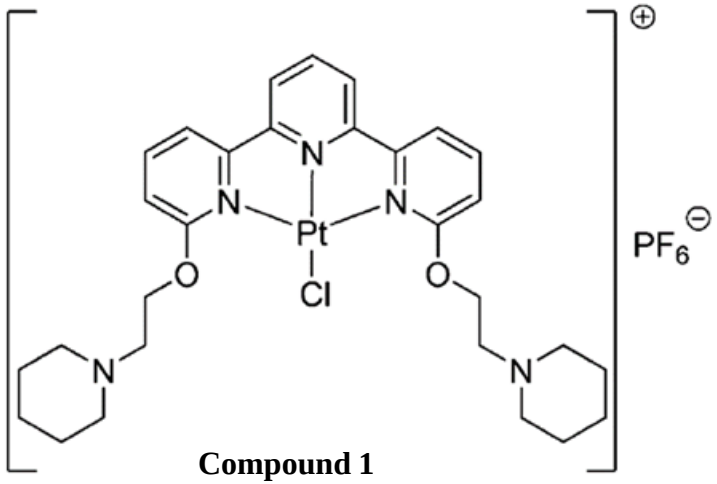
78 $4f^{14}5d^{9}6s^{1}$

Pt

Platinum

195.085

PLATINUM-based compounds



Compound 1

- New Pt(II)–terpyridine compound bearing two piperidine substituents in positions 2 and 2′
- Cytotoxic toward cancerous (U2OS and SH-SY5Y) and proliferating NIH 3T3 cell lines
- Able to induce cell death through necrosis

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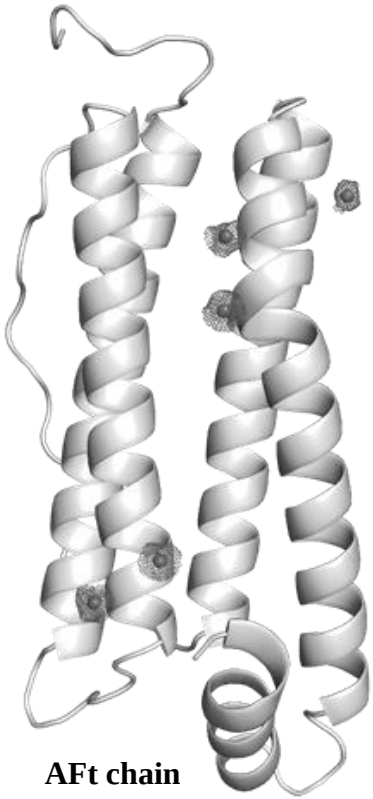
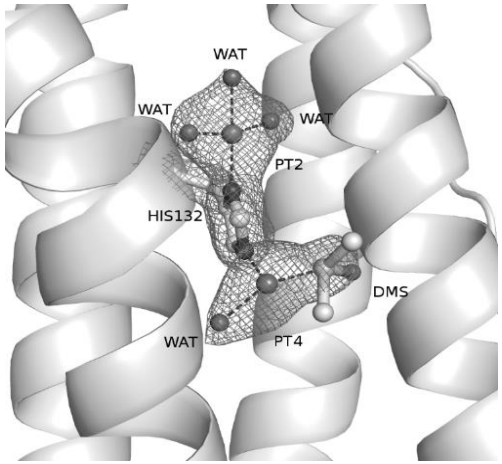
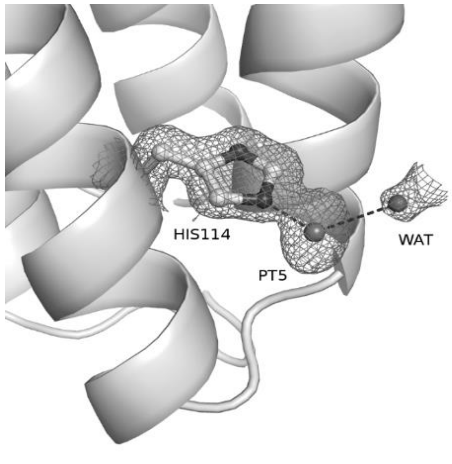
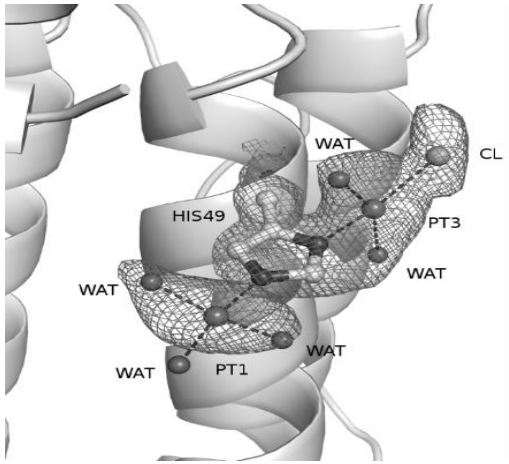
X-ray structure solution and refinement



AFt chain







- ✓ Data indicate the existence of five Pt(II) binding sites, close to the side chains of His49 and His132, and close to the side chain of His114

- ✓ Although Pt ligands are difficult to assign due to the limited occupancy (between 0.20 and 0.40), an attempt to complete the metal coordination spheres has been carried out by interpreting the electron density map with solvent molecules

Ferraro, G. *et al.* Nanomedicine (2018) 13: 2995–3007.

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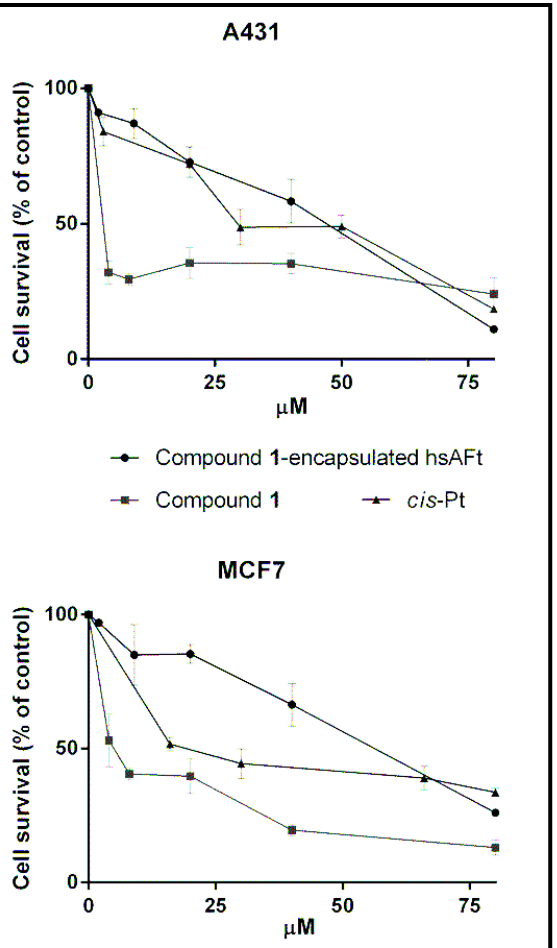
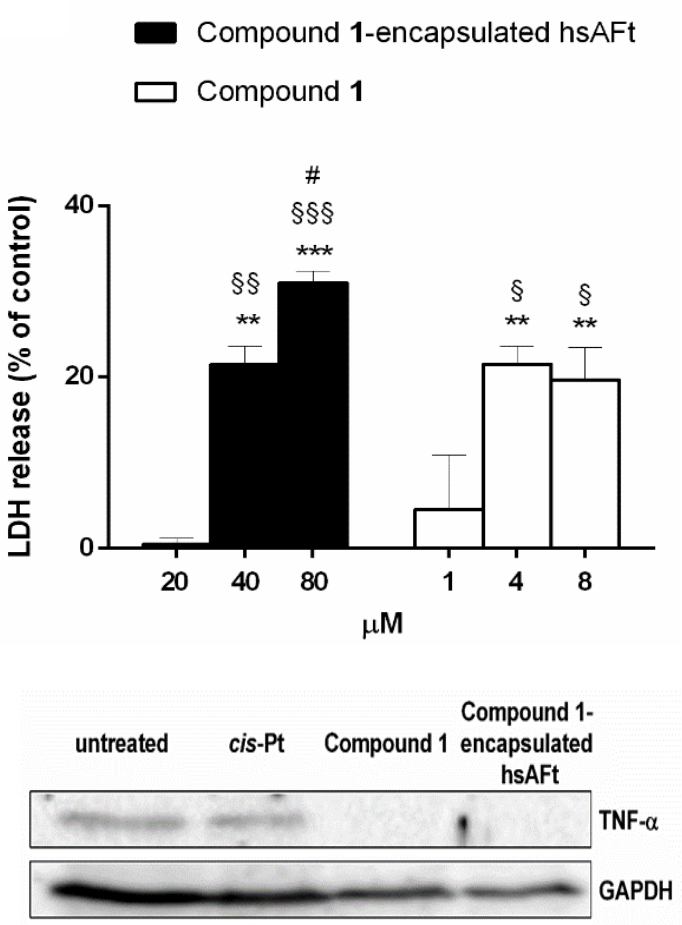


Pt(II)-AFt nanocomposite is less cytotoxic than the free compound, but it is more toxic than cisplatin at high concentrations



The Pt(II)-AFt nanocomposite triggers necrosis in cancer cells, as the free compound does

This suggests that the delivered drug is responsible for the biological properties of the Ft-Pt(II) nanocomposites and dictates the cell death mechanism

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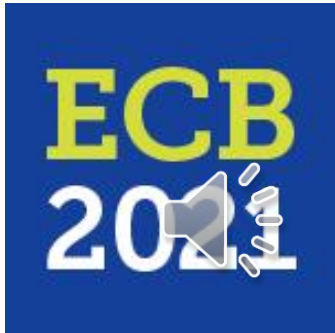
CONCLUSIONS

Well-characterized mono- and bi-metallic compounds of potential medicinal interest, belonging to different classes and containing different metals, have been selected and encapsulated within the ferritin nanocage, taking advantage of the assembly/disassembly encapsulation protocol.



These results allow to conclude that:

- The AFt nanocage reassembles upon the encapsulation protocol, with the protein that acquires its native conformation upon metal-based drug binding.

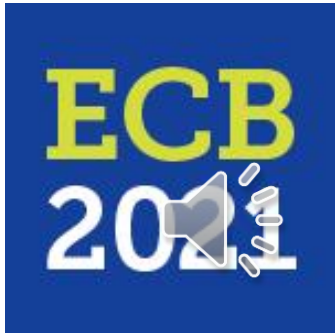
- The structure and the electrostatic potential of **the outer surface of AFt are basically not affected by the presence of the drug**. Drug-loaded AFt nanocages retain the physico-chemical features of the native protein, even upon drug encapsulation, confirming that this system can be used as a suitable nanocarrier.

- In the drug-loaded ferritins, **the binding sites are often located on the inner surface of the cage**. The metallodrug or metallodrug moieties can bind the side chains of AFt residues; but there are examples of compounds that are encapsulated within the cage, although they are not directly bound to protein residues.

- In all the characterized drug-loaded AFt systems there is a **significant amount of the metal compound in the bulk**. → These molecules could be the active species, i.e. the species responsible for the activity of the nanocomposites.

- **Ligand-free ferritin is non-toxic** either for normal or for cancer cell lines, confirming the biocompatibility of this nanocarrier. On the contrary, **the drug-loaded nanocarriers show moderate selectivity**, since they kill tumor cell lines at a lower concentration than that needed to kill normal cell lines.

- The cytotoxicity of the drug-encapsulated AFt depends mainly on the intrinsic properties of the encapsulated compounds, rather than on the structure of the ferritin obtained upon encapsulation.



Acknowledgments

Italian Association on Cancer Research

University of Florence

Prof. Luigi Messori

University of Naples Federico II

Prof. Antonello Merlino

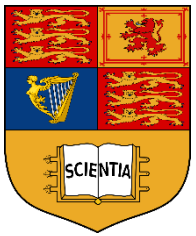
Prof. Daria M. Monti

Dr. Paola Imbimbo

University of Pisa

Dr. Alessandro Pratesi

Dr. Damiano Cirri



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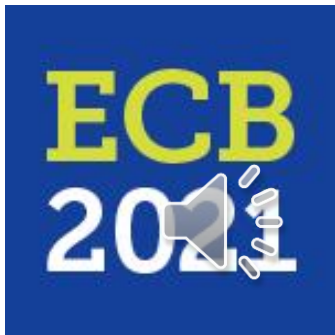


Thank you
for your
attention