

## **DIPARTIMENTO DI CHIMICA**

### **PROGETTO**

***La spettroscopia ESI MS, una tecnica di elezione per lo studio di addotti fra metallofarmaci e proteine.***

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**COLLABORATORI:** A. Casini, C. Gabbiani

Grazie ai suoi più recenti sviluppi, la spettrometria di massa di tipo ESI (Electrospray Ionisation Mass Spectrometry) rappresenta oggi un potente metodo di analisi delle interazioni di complessi metallici ad attività farmacologica con proteine. Infatti, oggi è possibile, tramite i metodi di ionizzazione “soft” tipici dell’ESI MS, trasferire intatto l’addotto metallo-proteina in fase gassosa. Ciò non è ovvio data l’intrinseca “fragilità” dei legami di coordinazione metallo-proteina. Questo metodo permette quindi di determinare la massa molecolare di un addotto con elevata accuratezza e di ottenere la sua completa caratterizzazione molecolare.

Tuttavia molto lavoro deve essere ancora fatto per l’ottimizzazione e la standardizzazione delle procedure sperimentali più adatte per studio di questi sistemi metallo-proteine. In effetti è riportata in letteratura una grande variabilità nella risposta del metodo ESI, in dipendenza di diversi fattori quali la natura della proteina, la natura del metallo e dei suoi leganti, le specifiche condizioni della soluzione (pH, tipo di buffer, forza ionica) nonché dei parametri strumentali.

Tramite questo la spettroscopia ESI MS sono stati analizzati, in collaborazione con il gruppo del CISM, gli addotti di ben noti complessi metallici ad attività antitumorale, cisplatino, transplatino, carboplatino e oxaliplatino, con la proteina modello citocromo c (cyt c). In una prima fase si sono messe a punto le condizioni sperimentali ottimali (tipo di buffer, pH, forza ionica, velocità di flusso dell’iniezione del campione, temperatura etc.) per visualizzare correttamente lo spettro della proteina. Quindi si è proceduto all’ottimizzazione di queste condizioni per i vari campioni complesso metallico/proteina. Fondamentale è stato garantire una

ionizzazione sufficientemente “soft” in modo da non distruggere gli eventuali addotti. I risultati ottenuti hanno permesso di evidenziare varie tipologie di interazione frammento metallico/proteina per ognuno dei complessi di platino selezionati. Ad esempio, se per il cisplatino è risultata chiara la presenza di mono- e bis-addotti con il citocromo, in cui il frammento legato è sempre  $[\text{Pt}(\text{NH}_3)_2]^{2+}$ , per il carboplatino, oltre al mono-addotto del frammento  $[\text{Pt}(\text{NH}_3)_2]^{2+}$ , si è osservato anche un mono-addotto della specie  $[\text{Pt}(\text{NH}_3)\text{CBD}]$  (CBD = cis-(1,1-cyclobutanedicarboxylato). Inoltre nel caso del carboplatino mancano completamente i bis-addotti del frammento platinato.

Questo lavoro di ricerca è stato riassunto nel seguente articolo:

“Exploring Metallodrug/Protein Interactions by ESI Mass Spectrometry: the Reaction of Anticancer Platinum Drugs with Horse Heart Cytochrome c.” Casini A., Gabbiani C., Mastrobuoni G., Messori L., Moneti G., Pieraccini G., Chem.Med.Chem., 2006 in press.

*Studi del meccanismo di azione di  
complessi metallici antitumorali  
e delle loro interazioni con proteine  
tramite tecniche di spettrometria di  
massa*

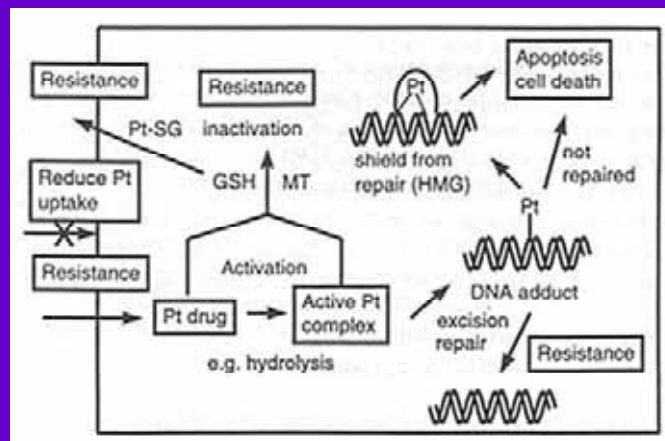
Luigi Messori  
“Gruppo Metalli in Medicina”  
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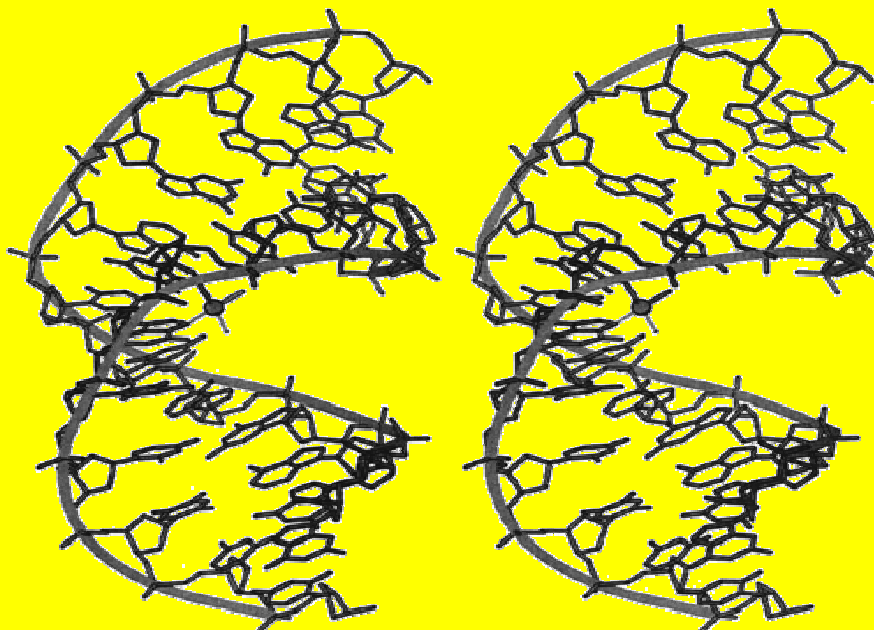
# METAL COMPLEXES AS ANTICANCER AGENTS

e.g. **Pt**, ... Ru, Sn, Pd, Au, Ti, Cu...

## THE MECHANISM OF ACTION OF CISPLATIN



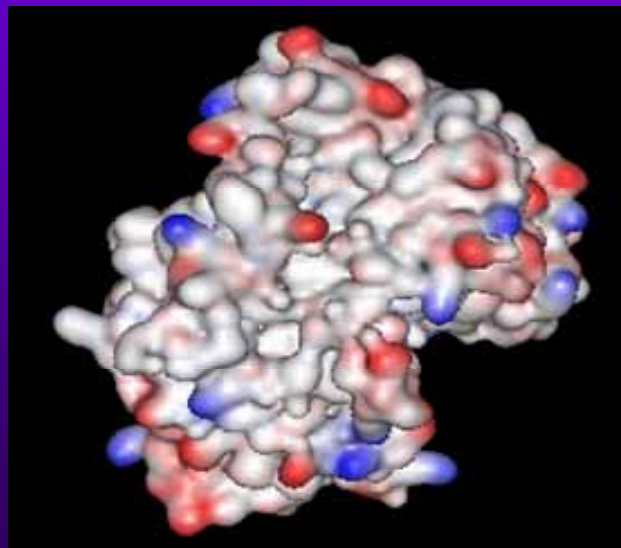
Crystal structure of an intrastrand adduct of cisplatin with a short DNA duplex  
Lippard et al. *Nature* 1995



**HOWEVER...**  
**PROTEINS ARE CRUCIALLY**  
**INVOLVED IN THE MECHANISM**  
**OF ACTION OF ANTICANCER**  
**METALLODRUGS**

**THE INTERACTIONS OF**  
**ANTICANCER METALLODRUGS**  
**WITH PROTEINS CANNOT BE**  
**NEGLECTED ANY MORE**

**PROTEINS**



Proteins are extremely complex molecules that offer a number of reactive sites for metallodrugs (e.g. his, cys, tyr, ser, asp, glu, met).

## CURRENT RESEARCH STRATEGIES

1. Assessing protein/metallodrug interactions on selected proteins; characterisation of the resulting protein-metallodrug adducts at the molecular level
2. Identification of the most important metal/protein adducts in cellular extracts (metalloproteomics)

## METHODOLOGIES FOR THE CHARACTERISATION OF METALLODRUG-PROTEIN ADDUCTS.

- Spectrophotometry
- Circular dichroism
- X-ray crystallography
  - NMR
  - ICP AES
- Equilibrium dialysis

# ESI-MS

- CHOICE OF APPROPRIATE SYSTEMS  
(SELECTION OF THE METALLODRUG  
AND OF THE PROTEIN)

- STANDARDIZATION OF THE  
EXPERIMENTAL CONDITIONS

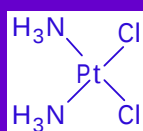
**Horse heart cytochrome c  
and platinum metallodrugs**

## Cytochrome *c* (horse heart)

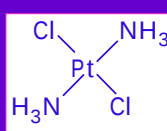


- 104 amino acids, Mw=12360 Da
- Basic pI=9.59
- Mainly helical
- Covalently bound heme
- Contains Cys, Met and His as possible metal complexes binding sites

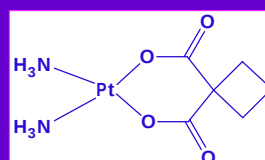
## Selected Platinum Compounds



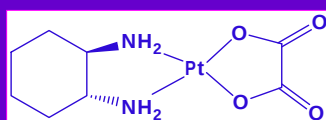
Cisplatin



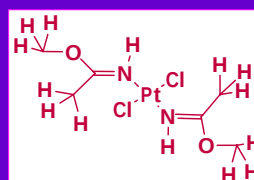
Transplatin



Carboplatin



Oxaliplatin

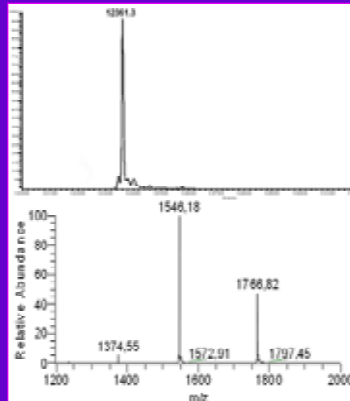


Trans-EE

## ESI - MS of cytochrome c

LTQ linear ion trap  
(Thermo, San Jose,  
California), equipped with a  
conventional ESI source.

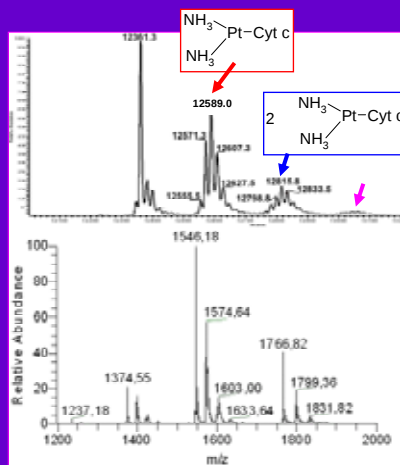
- Spray voltage = 3,5 kV
- Capillary voltage = 40 V
- Capillary temperature = 353 K
- Flow rate = 3  $\mu$ l/min.



Samples were prepared in  $(\text{NH}_4)_2\text{CO}_3$  buffer 25 mM, pH 7.4. Platinum to protein ratios was 3:1. Reaction mixtures were incubated for 72 h at 37 °C. Samples were extensively ultrafiltered in order to remove the unbound platinum complex.

- Under the non-denaturing experimental conditions used in this study, cytochrome c shows a spectral pattern that is dominated by the +8 multicharged species

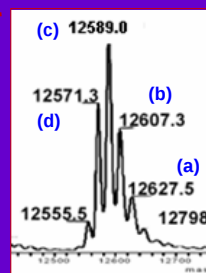
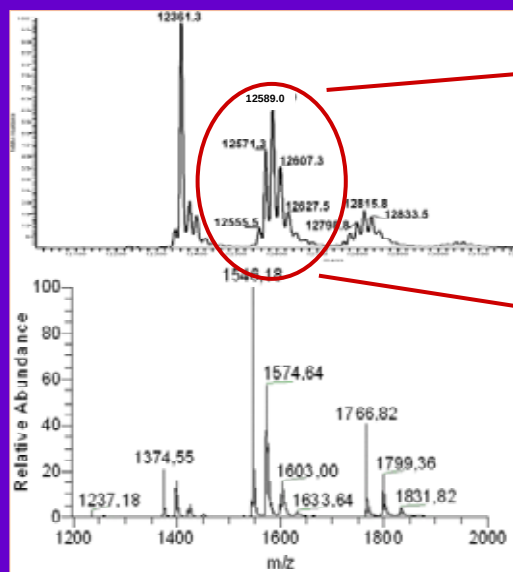
## Cisplatin



- The deconvoluted ESI MS spectrum of the cisplatin-derivative shows an intense multiplet centered at 12589 m/z that corresponds to an adduct in which a single  $[\text{Pt}(\text{NH}_3)_2]^{2+}$  fragment is coordinated to the native holoprotein.

- An additional multiplet is observed around 12816 m/z, corresponding to a 2:1 platinum-protein stoichiometry.

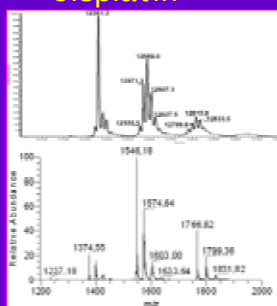
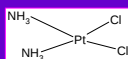
- Some additional weak signals, that are observed around 13062 m/z, might be assigned to an adduct with a 3:1 Pt/protein ratio.



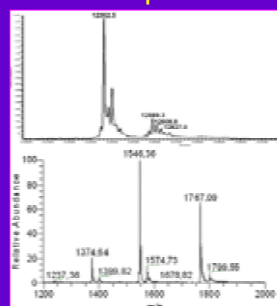
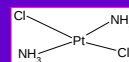
- (a)  $[\text{Pt}(\text{NH}_3)_2\text{Cl}]^+$   
 (b)  $[\text{Pt}(\text{NH}_3)_2\text{H}_2\text{O}]^{2+}$   
 (c)  $[\text{Pt}(\text{NH}_3)_3]^{2+}$   
 (d)  $[\text{Pt}(\text{NH}_3)]^{2+}$

Gibson, D.; Costello, C.E. *Eur. Mass Spectrom.* 1999, 5, 501.

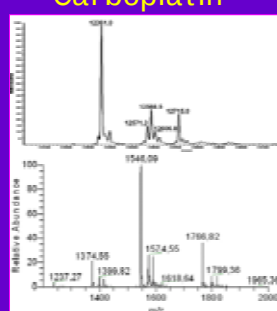
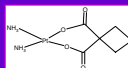
### Cisplatin



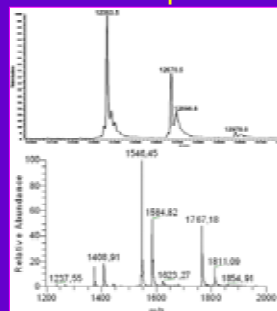
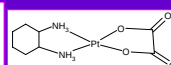
### Transplatin



### Carboplatin



### Oxaliplatin

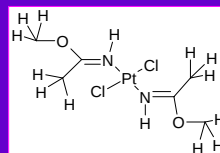
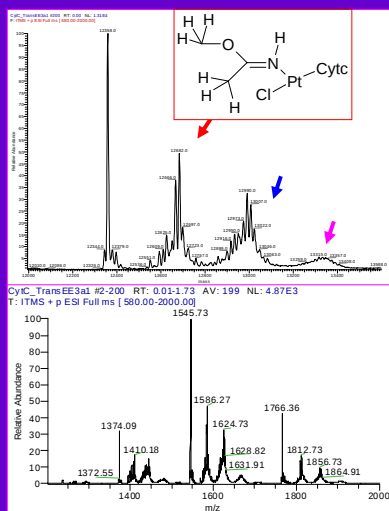


## ESI-MS RESULTS

- Predominant formation of monoplatinated adducts.
- We propose that **Met 65** represents the preferential binding site for platinum drugs while the imidazole groups of His 26 and His 33 may provide additional binding sites.
- UV-Visible spectra of the Pt-adducts pointed out that the protein is stable in its oxidised state. The protein chromophore is virtually unaffected by the interaction with the metal complex.

*Casini et al. ChemMedChem, in the press*

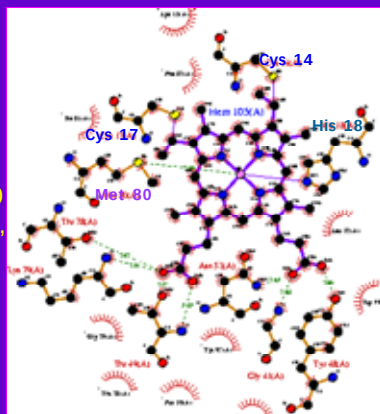
## Trans-EE



- A peak is observed at 12670 m/z that might well correspond to binding to cytochrome c of a  $[\text{PtCl}(\text{iminoether})]^+$  moiety.
- The peak of a 2:1 adduct, in which two fragments of the above kind are associated to the protein, is also observed at 12980 m/z.
- The intensity of the peak corresponding to the tri-adduct is higher than it has been detected in the other platinum derivatives.

## Possible binding sites for metal complexes on cytochrome c

•POSSIBLE  
ANCHORING SITES:  
Two met: Met 65, Met 80  
Three his: His 18, His 26,  
His 33  
Two cys: Cys 14, Cys 17



•FREE ANCHORING  
SITES:  
Met 65  
His 26, His 33

## PERSPECTIVES

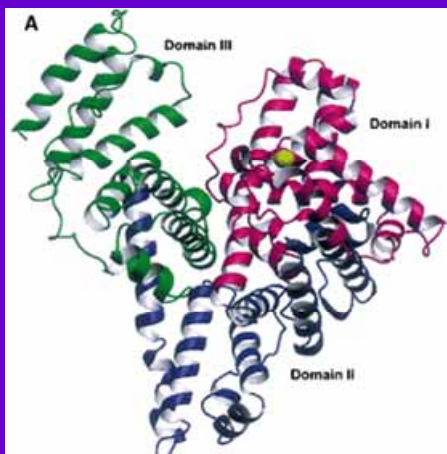
- ▶ Characterisation of other metallodrug-model protein adducts (e.g. Au and Ru metallodrugs)
- ▶ Characterisation of metal adduct with larger proteins e.g serum albumin, serum transferrin, thioredoxin reductase
- ▶ Metalloproteomics studies

## Human Serum Transferrin

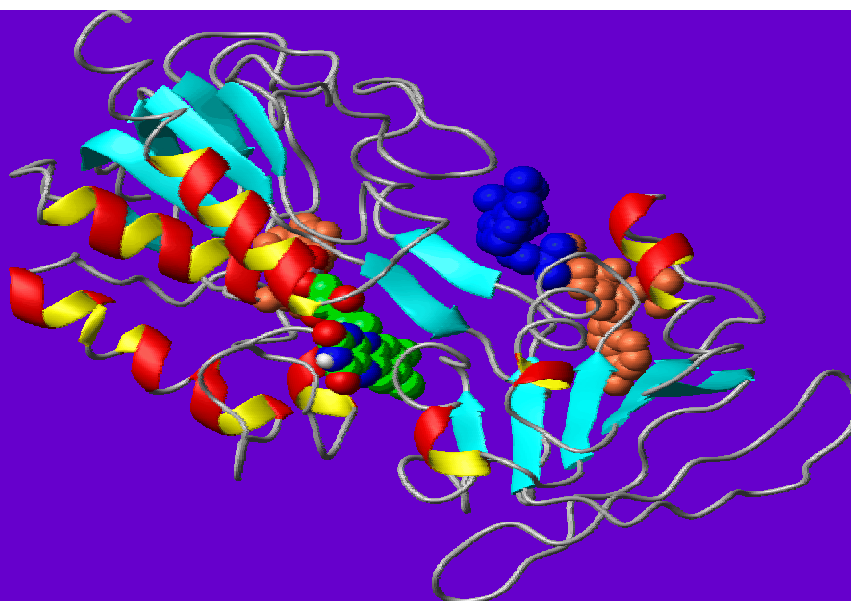


- Single chain
- 679 amino acids, 79.57 kD.
- Two lobes, each bearing a nearly identical metal binding site.
- Binding of metals is accompanied by simultaneous binding of the synergistic anion carbonate.

## Human Serum Albumin



- 586 amino acids, 66.4 kD
- Heart-shaped tertiary structure
- 28  $\alpha$ -helical regions
- 17 disulfide bonds
- Not a glycoprotein
- Nearly mM concentration in the plasma



**THIOREDOXIN REDUCTASE, A SELENOENZYME:  
A PROBABLE TARGET FOR METALLODRUGS**

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University of Bari



COST D20  
"Impact of Serum Proteins on  
Biodistribution, Metabolism  
and Tumour Targeting of  
Anticancer Metal Complexes"

Ente Cassa di Risparmio  
di Firenze

