Heterobimetallic complexes for theranostic applications
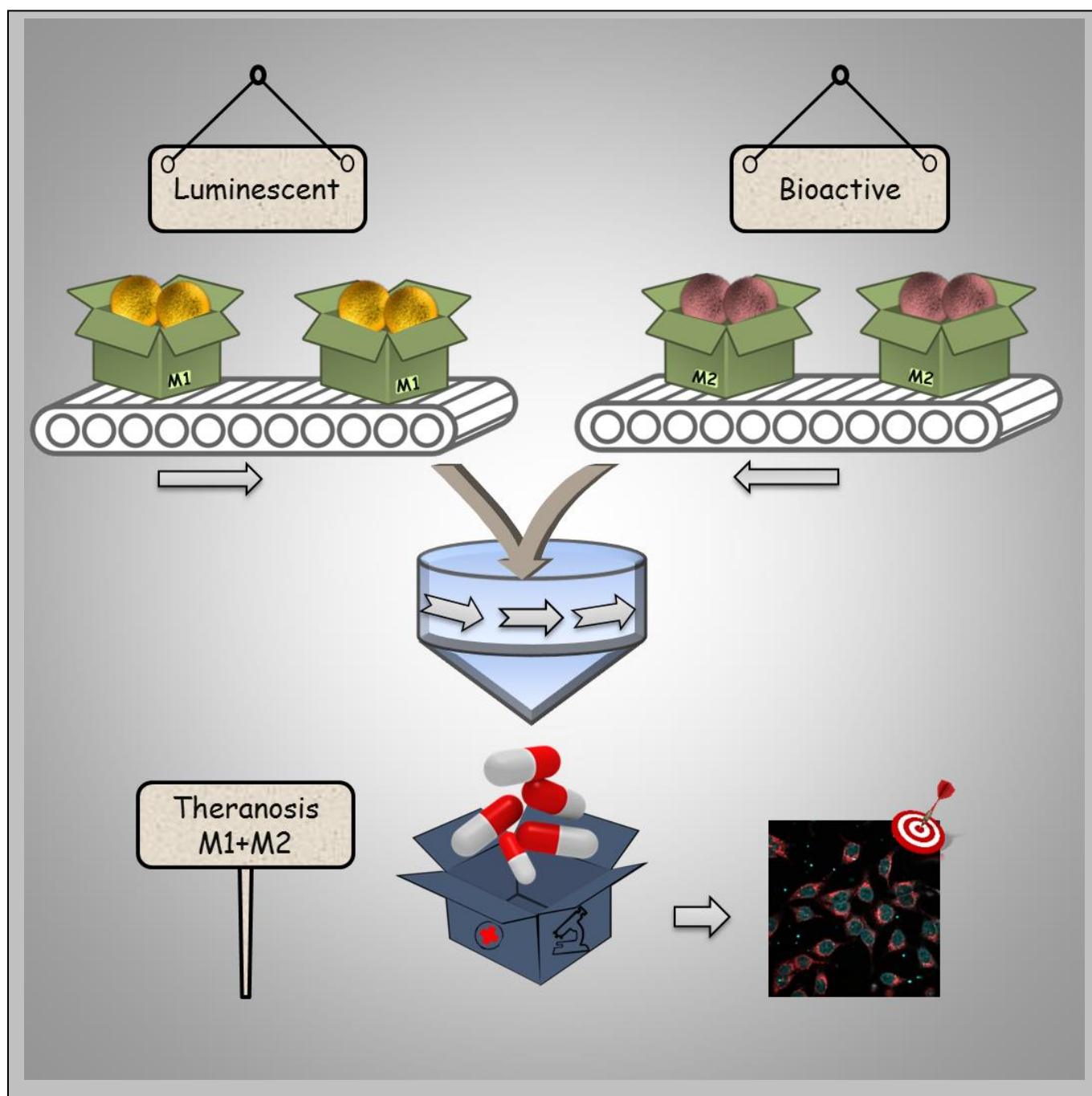
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Abstract: The design of more efficient anticancer drugs requires a deeper understanding of their biodistribution and mechanism of action. Cell imaging agents could help to gain insight into biological processes and, consequently, the best strategy for attaining suitable scaffolds in which both, biological and imaging properties are maximized. A new concept arises in this field which is the combination of two metal fragments as collaborative partners to provide the precise emissive properties to visualize the cell as well as the optimum cytotoxic activity to build more potent and selective chemotherapeutic agents.

Metallic drugs

Metal-based drugs have been used for the treatment of numerous diseases since the last century and represent important tools in contemporary medicine.[1] Gold salts were tested against tuberculosis and were employed as antirheumatics from the beginning of 20th century[2] but it is only with the discovery of cisplatin,[3] as one of the most powerful chemotherapeutic agents against ovarian and testicular cancer, that the interest for new metal-based therapeutic drugs starts. In particular, other platinum(II), ruthenium(II/III) and gold(I/III) based drugs, such as oxaliplatin, NAMI-A, KP1019, RAPTA-T or auranofin (Figure 1), have been pioneer compounds for the use of their respective metal in cancer therapy.[4] Although the biological target for platinum complexes such as cisplatin is the DNA, other platinum-based complexes and metals and have been probed to possess affinity for different biological targets which enable to reach a greater number of tumors. Thus, contrary to the presumed DNA-damage response mechanism of oxaliplatin, it was recently found that this platinum drug kills cells by inducing ribosome biogenesis stress.[5] In the same way, the main biological target of Au(I) and Au(III) complexes has been identified as the inhibition of the enzyme thioredoxine reductase (TrxR) which is overexpressed in a variety of cancer cells,[6] whereas ruthenium complexes exert anticancer effects through several targets including the inhibition of metastasis or the interaction with DNA or proteins.[7]
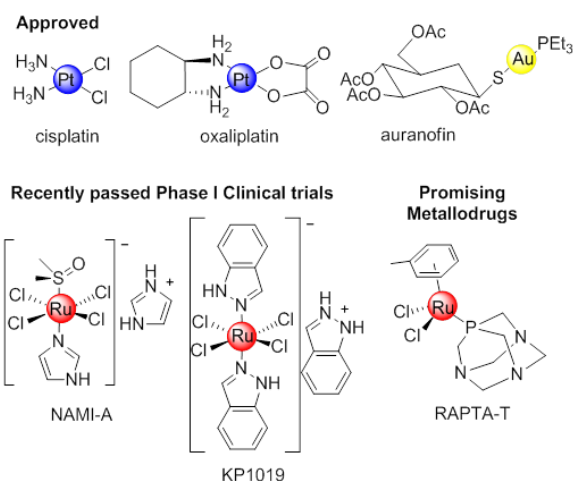

Figure 1. Metallic drugs already approved and in clinical trials

Additionally, it has been proved that the presence of two metallic fragments exerts a cooperative effect in the cytotoxic activity and properties of the final complexes. Moreover, they are often prone to overcome cellular resistance because of the possibility of combining the different intrinsic mechanism of action of each metallic fragment. Examples of this are illustrated in Figure 2, where normally the coordination sphere of the metals already emulates a known bioactive metallic environment, such as for instance Ru(II) as RAPTA,[8] Pt(II) as cisplatin,[9] titanium as titanocene,[10] and gold(I) as auranofin.[11]
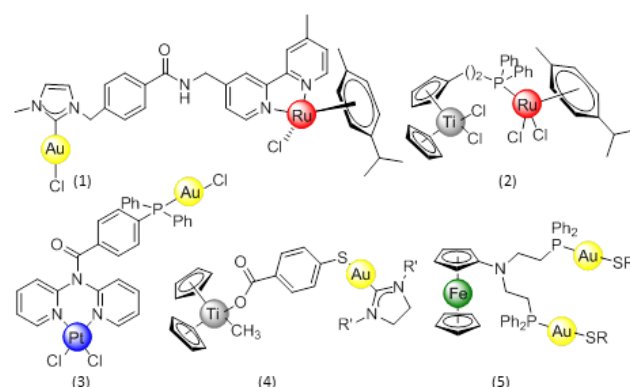

Figure 2. Bioactive heterobimetallic complexes.

Cell imaging agents

The design of more efficient anticancer drugs requires a deeper understanding of their biological interplay. Such demand is reflected by the great amount of technological advances that are becoming everyday available in the area of cell imaging. Fluorescence microscopy seems to play a key role to continue providing more hints in the development of new drugs. Its sensitive detection allows visualizing single molecules together with the possibility of monitoring rapidly changing events, which makes this technique as the ideal tool to reveal ground-breaking information about the mechanism of action of multiple drugs.

The idea of using fluorescence microscopy to visualize biomaterials relays in the availability of having molecules with the suitable photophysical properties that can be used as light-bulbs inside the cells. Although, traditionally organic fluorophores have dominated the area of cell imaging, in the last

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decade metal-based cell imaging agents have emerged due to their extraordinary photophysical properties. In particular, lanthanide compounds offer a series of advantages over organic chromophores in terms of Stokes' shifts and luminescence lifetimes.[12] Emission from lanthanide ions involves intra-configurational 4f–4f transitions, which are orbitally-forbidden transitions, so the excited state lifetime is long enough (µs–ms) to allow the use of time resolved techniques. Their emission patterns are easily recognizable and they are not affected by the surrounding environment. The major contributor to cell imaging applications are Tb(III) and Eu(III) complexes, which emit in the green and red regions of the spectrum respectively. As result of the orbitally-forbidden 4f–4f transition, the extinction coefficient of the lanthanides ion is very low. Therefore, a chromophore, often called as antenna, needs to be located within the surroundings of the lanthanide ion to ensure an optimum sensitization of the lanthanide. Particular care should be taken in the selection of the antenna, as the spectral overlap between the donor state of the chromophore and the absorption of the lanthanide ion is essential. In addition, the overlap with the emission of the lanthanide should be minimized to avoid a back transfer process and thus, a non-radiative deactivation pathway (Figure 3). Organic chromophores such as tetraazatriphenylenes, acridones, azaxanthones derivatives are wildly used as sensitizers for europium and terbium luminescence.[13] On top of that, saturating the inner coordination sphere of the lanthanide ion will also prevent the deactivation of the excited state. Typically, polyaminocarboxylate, β-diketonates or chromophoric chelates are used for such purpose. Several research groups as those headed by Parker, Bünzli, Yuan or Miller have demonstrated the application of Eu(III) and Tb(III) species in cell imaging.[14]
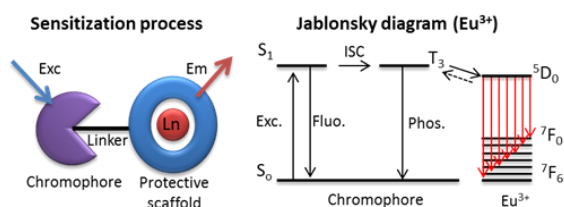

Figure 3. Sensitization process in lanthanide complexes.

In addition to those, transition d^6 metal complexes have also great potential as cell imaging agents. Their extraordinary photophysical properties,[15] i.e. ^{3}MLCT species with large Stokes shifts, long lifetimes and good quantum yields, have led to their successful application in fluorescent microscopy as cell imaging agents.[16] Moreover, their kinetic inertness due to the low-spin octahedral d^6 character confers these species with a low rate of ligand exchange, which is crucial in order to modulate the toxicity of heavy metal ions. The archetypal system for bioimaging applications are $[Re(N^\wedge N)(CO)_3(L)]^{0/+}$, $[Ru(N^\wedge N)_3]^{2+}$, $[Os(N^\wedge N)_3]^{2+}$ and $[Ir(N^\wedge C)_2(N^\wedge N)]^+$, where $(N^\wedge N)$ represents a bisimine derivative, $(N^\wedge C)$ a cyclometalated derivatives and L is a halogen atom or pyridine derivative (Figure 4). In the case of Re(I) derivatives, the specific phosphorescence process is generally due to ^{3}MLCT transitions and, specifically, a Re(dπ) → N^N(π*) transition. Therefore, tuning the biological activity, while the emissive properties are retained is possible with structural variations of L. Coogan and coworkers, were the pioneer in using these type of Re(I) complexes as cell imaging agents in 2007.[17] Since then, research groups such as those headed by Lo, Pope and lately by our research group have largely

contributed to widen the scope of Re(I) derivatives in fluorescence microscopy.[18] In contrast to Re(I) complexes where the unique bisimine is the only ligand that contributes to the ^{3}MLCT emission, the orbital combination of three diamine ligands of the Ru(II) and Os(II) species provides the ^{3}MLCT emission. Therefore, the nature of all of the chelate ligands as well as their functionalization modulates the luminescence.[19] Both of them share many photophysical properties. Ruthenium complexes have been long time studied as DNA light switch intercalators, oxygen sensors,[20] and lately as cell imaging probes.[21] However, Os(II) polypyridyl complexes have been only considered to bear biological applications very recently as a potential cell imaging agent by Keyes and coworkers.[22] In the case of cyclometallated Ir(III) complexes, their emission process is mainly due to a ^{3}MLCT process, although intraligand (^{3}IL), ligand-to-ligand (^{3}LLCT) and sigma-bond-to-ligand-charge-transfer (SBLCT) could also influence the luminescence. Their HOMO, largely localized on the metal and metallated aryl units and their LUMO on the pyridyl units, allow their energy levels to be tune independently and thus, a facile color tuning. Lo and co-workers[23] have been also pioneers in much of the work concerning bioconjugated cyclometallated Ir(III) complexes in cell imaging, although the first application as cell imaging agent came by Li and coworker using a pair of relatively simple cyclometallated species.[24]
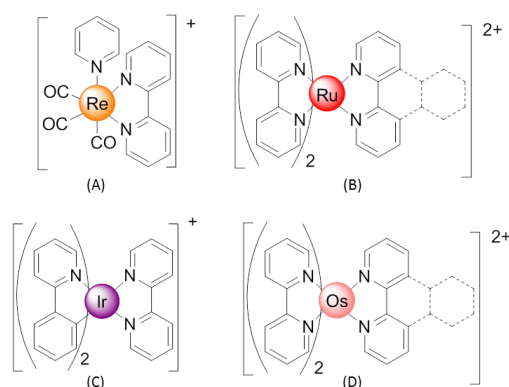

Figure 4. d^6 metal complexes as cell imaging agents.

At this stage it might be worth mentioning that some d^6 metal complexes have been proven to be phototoxic by either ligand dissociation or $^1O_2$ generation.[25] Therefore, a thoughtful ligand design is essential to tune or avoid undesirable outcomes. For instance, in the case of rhenium tricarbonyl polypyridine complexes, which are normally used for cell imaging proposes, could be transformed into a potent phototoxic complex by introducing 2-(2'-pyridyl)indolato derivatives as the bisimine ligand,[26] or by replacing it with tridentate derivatives.[27] For cyclometalated Ir(III) complexes, the generation of singlet oxygen have been observed for a wide range of derivatives, so it should be always considered as a potential cytotoxic component to be add to the overall behavior.[28]

Optical theranostic agents

Finding luminescent drugs that could be used with optical visualization techniques such as fluorescence microscopy is not common. In fact, none of the metal-drugs described previously (cisplatin, Auranofin, NAMI-A, etc.) bears the optimal optical

properties for being used within this technique. Therefore, an elegant approach to overcome such problem would be the combination of a visualization agent, i.e. and optical agent, with a selected therapeutic. Such bifunctional bioprobe, known as an optical theranostic or a trackable agent, would be able to provide relevant information regarding its biological interplay (Figure 5). The new knowledge could provide a major impact in medicine allowing the delivery of more efficient drug through a rationalized design.
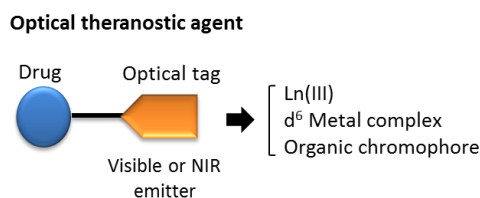
Optical theranostic agent

Figure 5. Design of an optical theranostic agents

Despite the great success of several metallodrugs as chemotherapeutic agents, the pharmaceutical industry is still reticent to incorporate metals within their designs. High toxicity, low aquo-solubility and further elimination issues are often used as justification not to give metal complexes a chance. However, we firmly believe that heterobimetallic complexes, as optical theranostic agents, represent a novel benchmark for delivering substantial advances in the frontier of knowledge. They will allow a better understanding of the interplay of anticancer drugs with biomolecules, which will ultimately help to the rational design of the next generation of metallodrugs. Additionally, metal complexes have a huge amount of variables to be modified that allows to fine-tune their properties as water solubility, stability or biodistribution. Organometallic probes may also represent a significant advance in tracking specific enzymes or proteins as they can be targeted after a specific functionalization of the luminescent probes.

The design of these metallic theranostic agents can be approached in two different ways:

   a) Introduction of an organic fluorophore in the skeleton of a metal-based therapeutic agent.
   b) Combining the properties of two different metal fragments, one for the imaging and the other for the therapy

Organic fluorophores such as acridine, anthracene, naphthalimide, coumarine and BODIPY have been traditionally used as optical probes for the development of trackable therapeutic agents. Despite their good commercial availability and relatively simple chemistry to be incorporated into the metal complexes, their photophysical properties are not always ideal for bioimaging purposes, where tissue light penetration is a handicap. Moreover, depending on the metallic drug couple with the fluorophore, the emission of the final complexes could be quenched via either a photoinduced electron transfer or by desexcitation of the triplet excited state.[29] Figure 6 shows some examples of different metallodrugs tethered with organic chromophores, which are the luminophores most studied so

far.[30]

Figure 6. Organic based luminescent bioactive metal complexes. [30]

From our perspective, designing drugs formed by two metallic fragments that are able to bring together emissive and therapeutic properties might result in a smarter approach that could be a turning point. As commented before, in some cases, combination of two metallic cores was proven to promote a synergy effect and thus increasing the therapeutic scope. Additionally, bimetallic species is likely to provide new sites for fine-tuning the photophysical properties and to adjust them to the optimal therapeutic window.

Therefore, this concept article will be limited to deal with trackable therapeutic agents that can be used in fluorescence microscopy, i.e. optical theranostic probes, and specifically in an interesting approach based on the use of two different metal fragments to provide in one side the emissive properties and in the other side the biological activity. These bimetallic theranostic agents may allow the development of more suitable and efficient optical metallodrugs able to be used in both diagnosis and therapy.

Heterobimetallic theranostic agents.

With this idea in mind, we pioneered the first bimetallic d^6-d^{10} optical trackable probe designed for such purpose, specifically Re(I)/Au(I) complexes, where the Re(I) fragment is providing the optical properties whereas the Au(I) provides the therapeutic activity.[31] The design of such complexes starts for the selection of the suitable fragments. Therefore, the tricarbonyl-bisiimine rhenium species were chosen as the emissive fragment and gold phosphine units as the biological scaffold. The key point to be considered is the selection of the ditopic ligand used to connect both metallic fragments. Taking into account that Re(I) complexes with pyridine as axial ligands have been used as cell imaging probes and that gold forms highly strong bonds with alkynyl ligands, the combination of both units could provide excellent platforms to be used as optical theranostic agents. Following this preliminary design, several pyridine or imidazole alkynyl ligands were chosen and the mononuclear rhenium(I) and the bimetallic rhenium-gold derivatives were prepared.[31] Any concern regarding the use of a potential toxic metal such as rhenium was withdrawn. Analysis of the cytotoxic activity of each metallic fragment separately and that of the heterobimetallic complex verified the low toxicity ability of the Re(I) core. Fluorescence microscopy revealed that, whereas the monometallic Re(I) species showed some general cytoplasmatic staining with a possible mitochondrial accumulation, the heterometallic Re(I)/Au(I) derivatives shifted from localizing in the mitochondria to the nucleus and nucleolus upon increasing the loading concentration (Figure 7). A completely different localization driving force might be implicated due to the presence of the bioactive gold fragment. Thus, the bioactive gold fragment is undoubtedly as the biological vector towards a still unidentified diana within the nucleus or nucleolus of the cells.
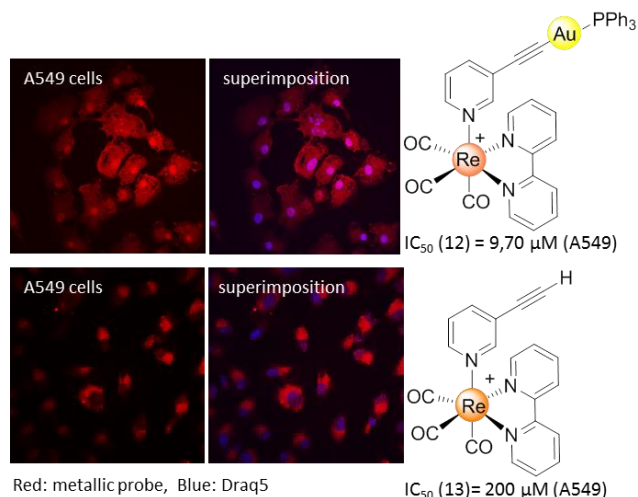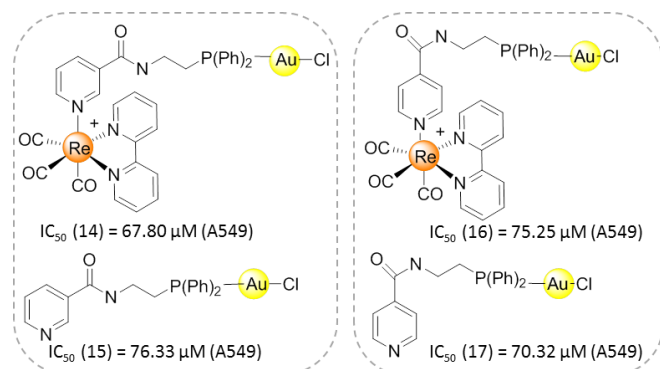
IC$_{50}$ (12) = 9,70 μM (A549)

IC$_{50}$ (13)= 200 μM (A549)

Red: metallic probe, Blue: Draq5
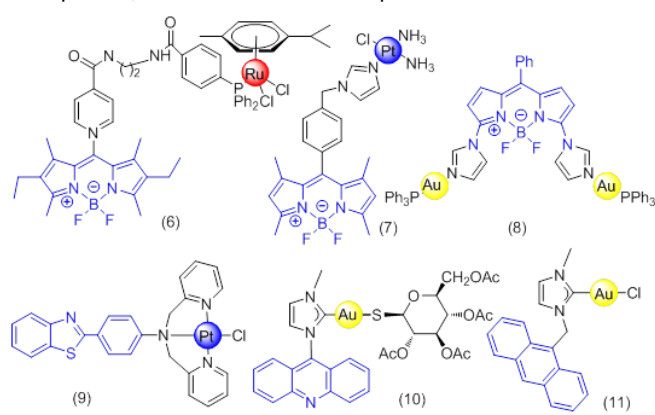
Figure 7. First trackable heterobimetallic probe. [31]

In general, interplay between both metal fragments seems to take place for the heterobimetallic species Re(I)/Au(I) reported. Thus, it could be postulated that the luminescent fragment barely alters the cytotoxicity of the conjugate. Figure 8 shows as the bioactive gold fragment 15 and 17, retain their cytotoxicity when coupled with the luminescent derivative to afford complexes 14 and 16 respectively.[32] Accordingly, the precursor rhenium species 13, is not cytotoxic until the bioactive gold derivative is coordinated, complex 12. On the contrary, the bioactive fragment might change the localization of the fluorophore at high concentrations as a consequence of its cytotoxic activity, as previously seen in Figure 7

Figure 8. cytotoxicity on Re(I)/Au(I) derivatives. [32]

IC$_{50}$ (14) = 67.80 μM (A549)

IC$_{50}$ (16) = 75.25 μM (A549)

IC$_{50}$ (15) = 76.33 μM (A549)

IC$_{50}$ (17) = 70.32 μM (A549)

Since then, and seeing the success of the proposed concept, "heterobimetallic species as optical theranostic agents", analogous examples were reported in the literature that highlight the potential of the approach. Thus, other explored examples included within the group of d^6-bifunctional probes are those heterobimetallic complexes using Ru(II) derivatives as luminophores. Specifically, Ru(II) complexes of the type [Ru(N^N)$_3$]$^{2+}$ coupled to either Au(I) or Ru(II) derivatives emulating the well-known bioactive auranofin or RAPTA-T

respectively,[33] or just combined with novel potential Au(I)/Ag(I)-NHC-based therapeutics.[34] Figure 9 exemplifies the importance of a thorough design to deliver an optimized bioprobe. In this case, substitution of bipyridine for a dipyridylamine in the ruthenium coordination sphere leads to the loss of luminescence intensity of the probe. However, the presence of the thioglucose as the gold ancillary ligand instead of chloride considerably increases the antiproliferative character. Regarding the biodistribution, they also offer a different pattern depending on the Ru(II) scaffold. Thus, complex 19 localizes in organelles within the nucleus, whereas complex 20 is distributed through the cytoplasm. Moreover both of them seem to enter the cell by active transport. Consequently, an in-depth knowledge of the different coordination spheres possibilities is essential because it is likely that the final bioprobe will combine the intrinsic properties of each metal fragment.
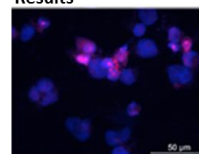
a) Heterobimetallic Ru(II)/Au(I)

(18) (19) (20)
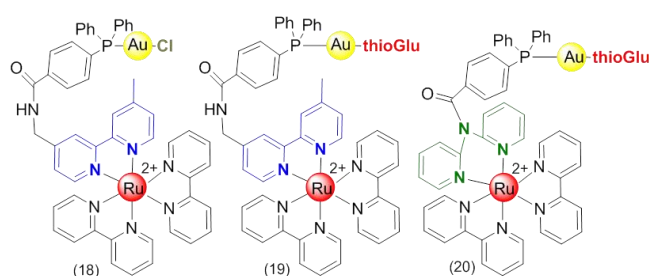
b) Heterobimetallic Ru(II)/Ru(II)
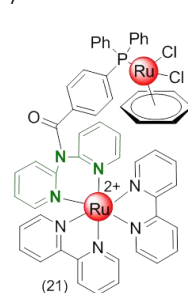
Results

Blue: DAPI, Pink: complex 19

Luminescence: 18 and 19 >>> 20 and 21
Cytotoxicity: 21 > 20 > 19 > 18
Localization: 19 → nucleus, 20 → Cytoplasm

(21)

Figure 9. Heterobimetallic trackable probes derived from luminescent Ru(II) complexes.[33]

Introducing NHCs within the structure of the scaffold of a potential metallodrug is a strategy that is gaining strength. They are good σ-donors delivering strong metal-carbene bonds and therefore, stable complexes in biological medium. Indeed, recent review articles have highlighted their interesting biological activities as potential antitumor metallodrugs.[35] Despite that heterobimetallic carbene derivatives have been already described in the literature, only a few examples deal with their biological properties, leaving the path free for future investigations in this area, Figure 10.[34,36]
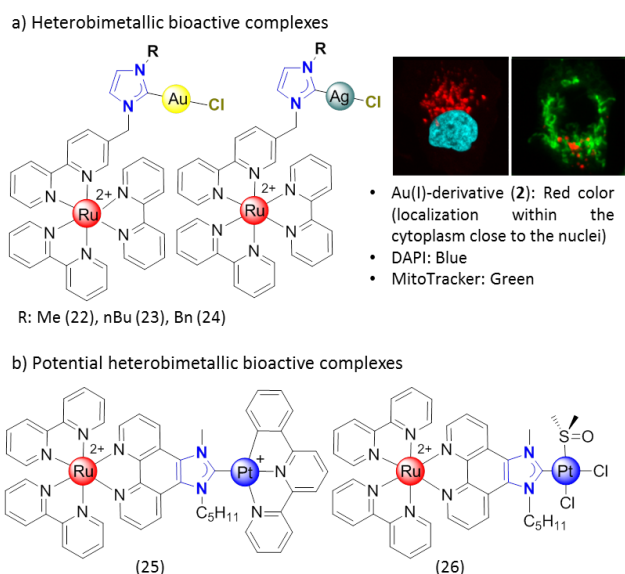
a) Heterobimetallic bioactive complexes

- Au(I)-derivative (**2**): Red color (localization within the cytoplasm close to the nuclei)
- DAPI: Blue
- MitoTracker: Green

R: Me (**22**), nBu (**23**), Bn (**24**)

b) Potential heterobimetallic bioactive complexes

Figure 10. Heterobimetallic complexes containing a NHC-fragment.[34,36]

Unfortunately, there are no examples in the literature dealing with the other well-known emissive d^6 metal fragments (i.e. Ir(III) and Os(II)) combined with bioactive metallic species. We believe that making an effort in studying heterobimetallic systems covering these metals is worth to be taken. The widely use of Ir(III) as cell imaging agent[37] would be an excellent starting point to outline a prototype and reach an optimized Ir(III)-based heterobimetallic teranostic agents. In the case of Os(II) species, a good strategy would be to relay in the photophysical similarities with that of Ru(II) species as only one example has been described dealing with Os(II) derivatives as cell imaging agents.[22]

Following the same idea lanthanide complexes could be also used as optical tags. Despite their interesting emissive properties in the biological field as well as their proven suitability as cell imaging agents,[38] only a couple of examples can be found in the literature where they are used as luminophores within a heterobimetallic theranostic agent. In 2015 Wong and coworkers[39] and one year later Patra and coworkers,[40] described the first examples of heterobimetallic Ln(III)/Pt(II) complexes that could be used as trackable and therapeutic probes. Specifically, Eu(III) and Tb(III) derivatives were chosen as red and green emitters, respectively. In both cases, the lanthanide ions were protected from the media with polyaminocarboxylic derivatives. Then, functionalization of either
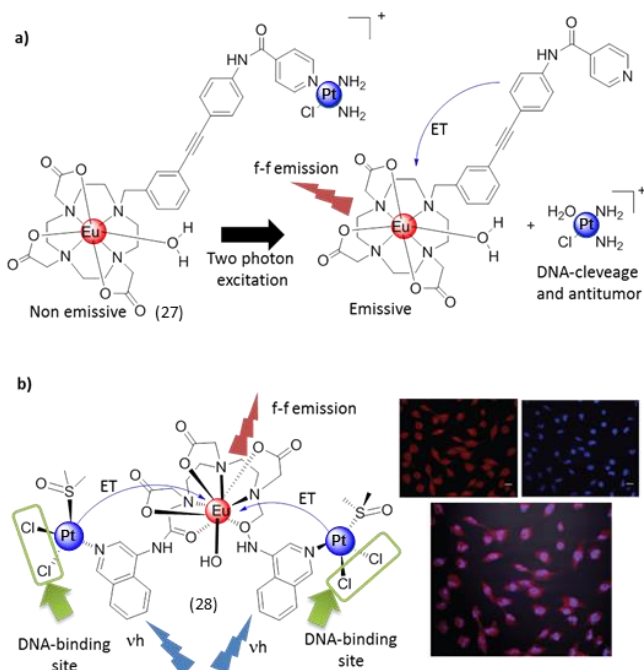
one or two of the carboxylic acid groups afforded the perfect linker to anchor the Pt(II) fragment, i.e. the therapeutic unit. The sensitization process was different for each probe. In the case of Wong and coworkers, a rigid chromophore was used as both bridge ligand and antenna, to *in situ* monitoring the photo-release of the antitumor Pt(II) complex, (Figure 11a). Instead, Patra's probe was sensitized via a ^{3}MLCT → f energy transfer process allowing a life tracking of the probe, while it is interacting with the DNA (Figure 11b).

Figure 11. Ln(III)-Pt(II) heterobimetallic complexes.

Advantages and optimization process

Apart from the emission efficiency (ϕ) and cytotoxic ability, there are additional premises that need to be tackle in order to build an effective trackable optical metal-based drug.

Selectivity stands up as one of the most important properties as it will bring higher efficiency and lower side effects of the drugs. Such selectivity could be achieved by tethering biological vectors as sugars, peptides or specific antibodies.[41] Additionally, selectivity could be also attained by director groups such as for instance *N*-hydroxysuccinimidyl ester or isothiocyanate, susceptible to react with primary amines, and iodoacetamide, maleimide and methylchloride prone to react with thiolate groups, Figure 12, as well as planar aromatic platforms which would tend to intercalate within the bases of DNA.[42] One of the advantages of using biological vectors over director groups to deal with selectivity is that these biomolecules will possibly provide additional *solubility* in biological media and *cell permeability,* which are also essential properties for a potential drug. In the same way, the gain of using heterobimetallic probes over monometallic or even organic compounds is that there are two different environments, i.e. two independent coordination spheres, in which a biovector could be tethered. In this way, the synthetic approach for the functionalization eases as vector group is not bound to be introduced in a specific scaffold. Moreover, structural modifications on organic chromophores could be demanding whereas the synthesis of most of the already mentioned metallic complexes is made by well-established synthetic procedures involving a stepwise introduction of ligands.[43] In addition, heterobimetallic complexes bear a higher flexibility when it comes to modulate solubility. Typically, organic platforms are quite lipophilic resulting in poor water solubility. However, formal charge of heterobimetallic complexes can be easily modulated with the appropriate ligand scaffolds or functional groups. Once again, modulation of the formal charge influences the *cellular permeability* ability, *internalization rate* and even the *uptake mechanism*.[44]
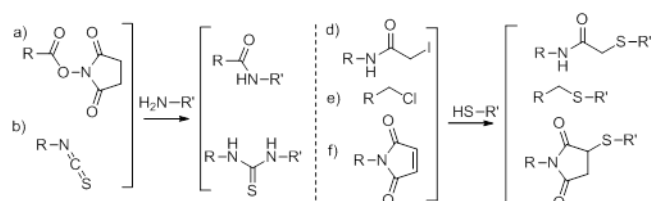

Figure 12. Director groups.[35a]

A clear advantage of using heterobimetallic complexes over other potential probes for theranosis, especially in comparison with organic scaffolds is related with the intrinsic photophysical properties of d^6 metal complexes and lanthanides. Typically their large Stokes shifts prevent from reabsorption processes to take place that could lead to emission self-quenching, their long lifetimes allow the use of time-gating techniques and in consequence autofluorescence of the sample could be avoided providing images with excellent signal/noise ratio. In addition, most of them offer the possibility of using long wavelength radiation (>400 nm) and emission within the therapeutic window (650-900 nm). Therefore, excitation of organic biomolecules within the sample is prevented and the sample will be less damaged. In the same way, being able to irradiate and visualize the sample using long wavelengths allows a higher tissue penetration, which could be very useful to take the leap from *in vitro* to *in vivo* preclinical models. [16d,37c,38] Besides, color tuning is normally easier in metal complexes than with organic chromophores, as their HOMO and LUMO orbitals are often localized in different parts of the molecule. This allows the modification of the energy levels independently being able to reach a wide of wavelengths across the spectrum.[45]

Theranostic agents might provide additional values to consider. They could be designed in a way that, not only would give us the knowledge on the location of the drug, but also additional information regarding its mechanism of action. Thus, switch on-off probes, or just probes able to change their photophysical behavior upon an external factor (pH, hypoxia, redox potential, presence of a specific biomolecule, complex, etc.) would be of great interest to reveal the depths of the responses of the human body to a specific drug. This strategy could renew the concept of trackable theranostic agents by adding a groundbreaking approach to theranosis. Few examples have been described in the literature where the luminescence of trackable metallodrugs is triggered by a specific factor. All of them are based on metallodrugs tethered with organic chromophores that either switches on upon reaching the target,[46] or upon detecting a change in the oxidation state of the metal center,[47] or just after the activation of the prodrugs takes place[48] or even when the cell enters in apoptosis.[46] There is a wide variety of combinations that can be made in this area or theranostic switchers, that would surely end up by providing valuable information onto the mechanism of action of metallodrugs. Despite their promising future, this new concept is still to be explored using heterobimetallic probes.

Conclusions and future directions

The development of more efficient and personalized drugs remains as one of the worldwide greatest challenges. The discovery and continual refinement of diagnostics and therapeutics is approached by numerous research groups in order to satisfy the present demand for early disease detection and better treatments. Within this frame, the development of optical theranostic agents emerges as a versatile method to rich this goal. The examples presented in this concept article clearly illustrate the great potential of heterobimetallic complexes as optical theranostic probes. Thus, although drug development is dominated by organic chemistry, the importance of inorganic

medicinal chemistry is beyond any doubt. From metallodrugs, such as cisplatin for cancer treatment or auranofin for rheumatoid arthritis, to commercial available metal-based optical probes used for cellular visualization (Eu-W8044, [Ru(Bipy)$_2$(5-NCS-Phen)](PF$_6$)$_2$, etc.) inorganic and organometallic complexes are definitely having a great impact in medicine. Their versatility in terms of oxidation state, geometry, charge or solubility provided an excellent source of building blocks that can be used to rationally deliver the desired trackable metallodrug. Only a few examples have been already described so far and has been seen the scope of applications that they offer. We firmly believe in the perspectives and possibilities to build novel metallodrugs based on two metal fragments, each of them perfectly tuned to rich the maxima effectiveness within their nature. Once they are brought together, a synergy effect would surely take place to maximize their optical and pharmacological effect.

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The design of more efficient drugs requires a deeper understanding of their biological distribution and mechanism of action. A smart approach would be the combination of two well-known metallic fragments, i.e. a luminescent and a bioactive fragment, able to generate a synergy between their intrinsic properties. Thus, it will be feasible to use non-invasive techniques such as fluorescence microscopy to reveal key information for futures advances in medicine.

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