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Luminescent conjugates between dinuclear rhenium complexes and 17 α -ethynylestradiol: synthesis, photophysical characterization, and cell imaging.

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Three new luminescent conjugates between the dinuclear Rhenium complexes and the estradiol, namely **E2-Re**, are described. The derivatives have the general formula $[\text{Re}_2(\mu\text{-Cl})_2(\text{CO})_6(\mu\text{-R-pydz-17}\alpha\text{-ethynylestradiol})]$ (R-pydz = functionalized 1,2-pyridazine), where the estradiol moiety is covalently bound to the β position of the pyridazine ligand. Different synthetic pathways are investigated, including the inverse-type [4+2] Diels Alder cycloaddition reaction between the electron poor 1,2,4,5-tetrazine and the 17 α -ethynylestradiol for the synthesis of **E2-Re1**. The three **E2-Re** conjugates are purified on silica gel and isolated in a spectroscopically pure form with moderate to good yields (28-50%). All the **E2-Re** conjugates are comprehensively characterized from the spectroscopic and photophysical point of view. Cellular internalization experiments on human MCF-7 and 231 cells are also reported, displaying interesting staining differences depending on the nature of the spacer linking the estradiol unit to the organometallic fragment. Furthermore, the suitability of these conjugates to stain also simple multicellular organisms, i.e. *Ciona intestinalis* embryos and larvae at different stages of development is here reported for the first time.

Introduction

Estrogens are essential hormones involved in many physiological and pathological (e.g. tumorigenesis) life processes of both adults and embryos, where they exert a fundamental role via specific molecular transducers (receptors). The critical importance of these hormones is demonstrated by the fact that an impairment of their functioning can cause severe adverse effects to the organism, in both humans (such as reduced fertility, carcinogenesis) and wild-life.¹ Therefore, the possibility to determine the estrogen receptor (ER) content of the cells is essential from different point of view.

In the past, the design of probes for these proteins has relied mainly on radioactive estrogen derivatives such as radioiodinated derivatives² or [F-18]-estrogens.³ ^{99m}Tc is also a readily available nuclide with highly desirable physical properties.⁴ Today, in most clinical and research studies, ER are detected by immunolabelling (i.e. by means of specific antibodies raised against the protein). Although this is certainly a well-established procedure, it can have some limitations such as for example species-specificity (i.e. an antibody raised against the human ERs hardly works on wild-life species). Furthermore, immunotechniques can be used only on "already known" ER, whereas the discovery and study of novel ER forms both in humans or in other animal species requires the use of indirect ligand-based methods, such as E2-labelled probes.⁵

The possibility of using luminescent probes for estradiol-binding proteins has also been explored. In particular a wide range of estradiol conjugates containing organic fluorophores have been reported,⁶ while the use of luminescent organo-transition metal complexes has not been completely explored. Actually only few mononuclear rhenium complexes⁷ and cyclometalated iridium (III) polypyridine estradiol conjugates have been reported,⁸ despite many of the photophysical properties of luminescent transition metal complexes make them good candidates for applications in cell imaging. Indeed, their emission characterized by large Stoke shift and long luminescent lifetime, allows differentiating their emission from the autofluorescence of the cell. Moreover, the excited states of organometallic complexes are particularly stable toward degradation, hampering the photobleaching and reducing their cytotoxicity. Further, different parameters such as charge, size and balance between lipophilicity, and water solubility of the complexes can be controlled in order to increase their cellular uptake. Therefore a range of structural analogs can be designed by this approach, with not demanding synthetic procedure and with the same photophysical properties, allowing to test the better probes.⁹

In this framework, in the last years we have developed a novel family of dinuclear tricarbonyl rhenium(I) derivatives (see Chart 1) containing a diazine ligand, which exhibit intense emission (photoluminescence quantum yields up to 0.5) in the range 550–620 nm, originating from triplet metal-to-ligand-charge transfer excited states ³MLCT.¹⁰ Some of them have been successfully used as luminescent probe for Peptido Nucleic Acids (PNAs) and tested in cell imaging experiments.¹¹ Moreover, the same complexes have been employed as new, simple, chemically robust, and easy-to-synthesize platform for different carbohydrate ligands in order to develop novel luminescent glycosylated materials.¹²

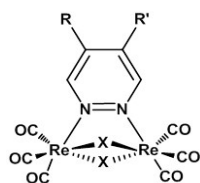


Chart 1 General structure of dinuclear Re(I)-complexes bearing a bridging 1,2-diazine ligands with different alkyl groups (R and R') and two ancillary anionic ligands (X = Cl, Br, I).

These interesting results have prompted us to synthesize three luminescent rhenium-estradiol conjugates (see Chart 2) and to test their localization inside the MCF-7 cells, which express the estradiol receptor.¹³ Furthermore, we designed experiments to evaluate, for the first time, the suitability of the rhenium conjugates also in simple multicellular organisms, i.e. *Ciona intestinalis* embryos at different stages of development.

The 17 α -ethynylestradiol has been selected as reagent for the synthesis of the new ligands, which are able to coordinate the dinuclear rhenium complex, giving luminescent estradiol conjugates. The nature and the length of the linker between the 17 α -estradiol and the luminescent organometallic core, together with the substituents on the diazine ligand, have been varied to tailor the desired conjugate properties, especially in terms of photophysical properties.

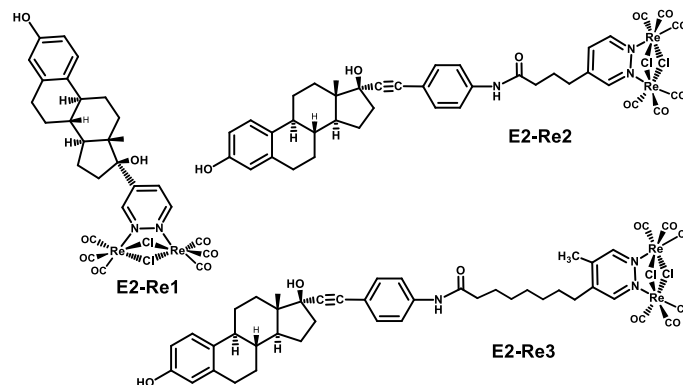


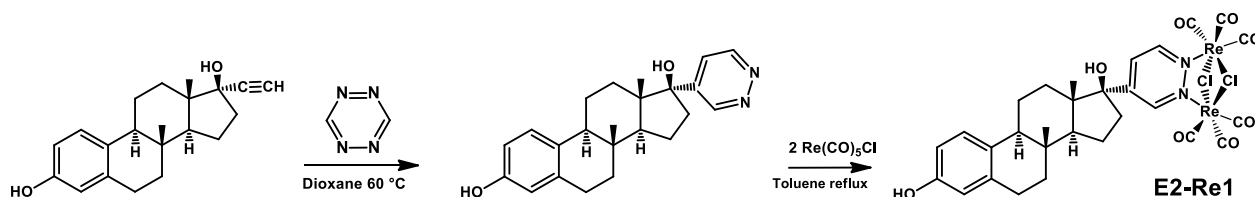
Chart 2 Molecular structure of the three E2-Re conjugates

Results and discussion

Synthesis.

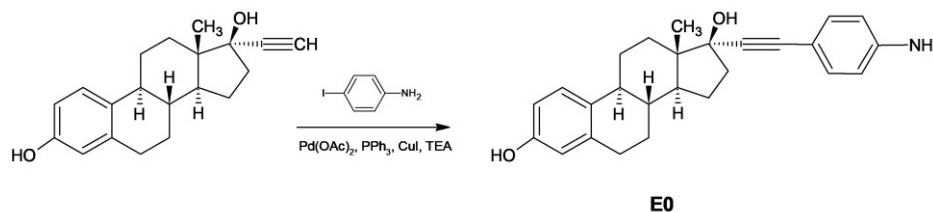
The design of the new Re-estradiol conjugates is based on previous works that clearly demonstrated that the absence of the 17 β -hydroxyl substituent considerably reduces any effective binding to the receptor site.¹⁴ Therefore, we have chosen to retain the 17 β -OH linkage and to bind the organometallic moiety to the 17 α position. Moreover, it has been demonstrated that the identity of the spacer linking the organometallic fragment to C(17) makes a marked difference, with a significant enhancement of the receptor binding affinities (RBA) when a rigid alkyl chain is introduced.¹⁵ Then, starting from the hormone precursor the 17 α -ethynylestradiol, two different synthetic routes have been carried out for the synthesis of the three novel Re-estradiol conjugates.

In particular, in the case of complex **E2-Re1**, the estradiol fragment has been directly linked to the pyridazine ring by means of the inverse-type [4+2] Diels Alder reaction between the electron poor 1,2,4,5-tetrazine and the 17 α -ethynylestradiol with N₂ loss (see Scheme 1) affording the corresponding 3-(17 α -estradiol)pyridazine **D1**. The corresponding dinuclear rhenium conjugate **E2-Re1** has been obtained by refluxing **D1** with two equivalents of Re(CO)₅Cl in toluene solution using the method previously reported.^{10a}



Scheme 1 Synthetic route of complex **E2-Re1**

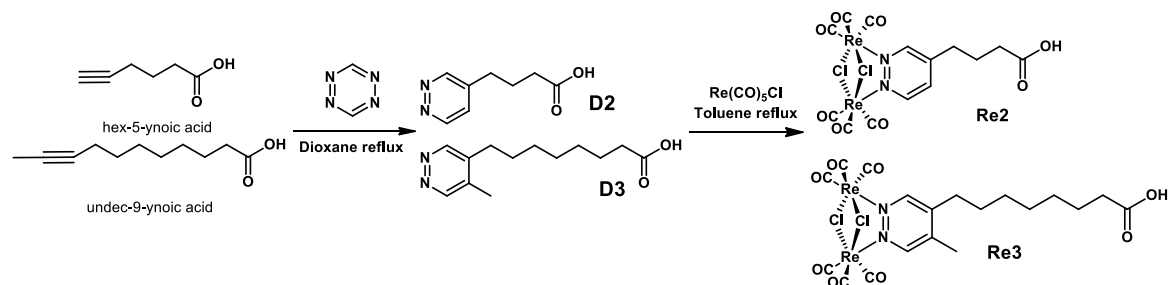
In the case of conjugates **E2-Re2** and **E2-Re3**, an alkyl spacer has been introduced between the organometallic fragment and the 17 α -ethynylestradiol, in order to increase the distance between the Re(I) complex and the 17 β -OH group, avoiding any steric repulsion between this functional group and the carbonyl groups on the two metal centres. For this purpose the 17 α -ethynylestradiol has been previously functionalized with a p-aniline by means a Sonogashira palladium-catalysed coupling reaction, between the 4-iodoaniline and the 17 α -ethynylestradiol, affording the 4-(17 α -ethynylestradiol)aniline precursor (**E0**, see Scheme 2).^{7b}



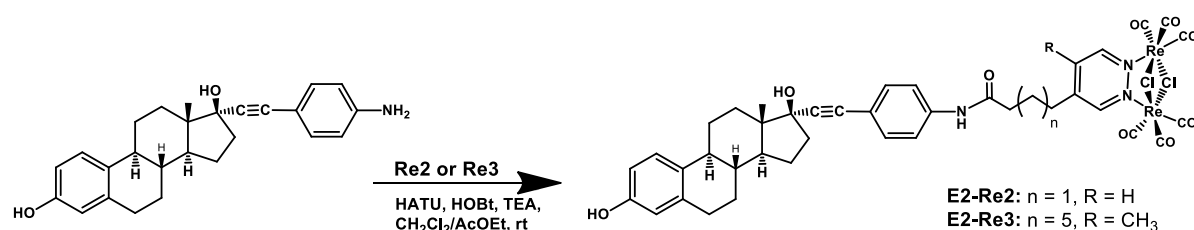
Scheme 2 Synthesis of the 4-(17-Ethynylestradiol)aniline (**E0**) precursor

Two dinuclear Rhenium complexes **Re2** and **Re3** (see Scheme 3), containing two different diazine ligands, namely 4-(pyridazin-4-yl)butanoic acid (**D2**, Scheme 3) and 8-(5-methylpyridazin-4-yl)octanoic acid (**D3**, Scheme 3), have been prepared following the previously reported synthetic method.^{10a,11a} Exploiting the reactivity of the carboxylic acid, both the complexes were further covalently linked to the estradiol precursor **E0** through the peptide coupling reaction in the presence of HATU and DIPEA at room temperature (see Scheme 4).

The three conjugates have been purified on silica gel and isolated in a spectroscopically pure form with moderate to good yields (28–50%).



Scheme 3 Synthesis of the two Re(I) complexes



Scheme 4 Synthesis of the Re-conjugates **E2-Re2** and **E2-Re3**

Photophysical Characterization.

The photophysical properties of the Re-estradiol derivatives **E2-Re1** - **E2-Re3** and of their parent complexes **Re2** and **Re3** have been studied in diluted (concentration of 1.0×10^{-5} M) both de-aerated toluene solutions and air-equilibrated $\text{H}_2\text{O}-\text{CH}_3\text{CN}$ solution at room temperature (the data are listed in Table 1).

At long wavelengths, the electronic absorption spectra of conjugates **E2-Re1-E2-Re3** displayed broad absorption band with maxima in the range 383–364 nm with a moderate intensity ($\epsilon \approx 0.86\text{--}0.95 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$). Such transition can be assigned to the spin allowed $d(\text{Re})-\pi^*(\text{diazine ring})^1\text{MLCT}$ band as typical of this class of complexes.¹⁰

The charge transfer character of this electronic transition is supported by: a) blue shift of the absorption and a red shift of the emission maxima, by increasing solvent polarity; b) modulation effect on the photophysical properties on varying the substituents on the diazine ligand. Indeed, complex **Re3**, and its corresponding estradiol conjugate **E2-Re3**, featuring an electron-richer diazine ligand, containing two alkyl substituents in the β positions, displayed a blue-shifted absorption band, with respect to the mono substituted complexes **E2-Re1**, **E2-Re2** and **Re2**. This is in agreement with the different energy level of the LUMO, mainly localized on the π^* of the pyridazine ligand, which is higher for the di-substituted pyridazine of **Re3** and **E2-Re3** than for the mono-alkylated ones. The conjugation of the estradiol moiety on the organometallic scaffold does not modify the absorption properties, as previously noted for the PNA-conjugates¹¹ and for the glycol-derivatives¹²

Table 1: Photophysical data for the **Re2** and **Re3** complexes and their related conjugates **E2-Re1**, **E2-Re2** and **E2-Re3** in ^a de-aerated toluene solution and ^bin air-equilibrated $\text{H}_2\text{O}-\text{CH}_3\text{CN}$ 1:1 solution, at 298 K.

Compound	λ_{abs} [nm] ^a	λ_{em} [nm] ^a	Φ^a	τ [μs] ^a	$k_f(\times 10^{-5})\text{s}^{-1}$	$k_{nr}(\times 10^{-5})\text{s}^{-1}$
E2-Re1	383	586 (608) ^b	0.19 (0.017) ^b	1.8 (0.280) ^b	1.05	4.50
E2-Re2	370	585 (607) ^b	0.18 (0.012) ^b	1.7 (0.220) ^b	1.06	4.82
E2-Re3	364	558 (580) ^b	0.40 (0.015) ^b	3.8 (0.270) ^b	1.05	1.58
Re2	370	586	0.12	1.6	0.75	5.50
Re3	364	555	0.55	4.7	1.17	0.96

Upon excitation in the range 440–450 nm at room temperature, in toluene solution, all the samples showed broad and featureless emission in the yellow-green region of the visible spectrum (see Fig. 1), which is assigned to the radiative decay of the ³MLCT excited state, in comparison with closely related complexes.^{10b,11} Similarly to what observed in the absorption spectra, complex **Re3** and its related steroid-conjugate **E2-Re3** displayed remarkably different emission features compared to derivatives **E2-Re1**, **E2-Re2** and the precursor **Re2**. In particular, **E2-Re3** and **Re3**, displayed a hypsochromic shifted emission, characterized by a higher and longer

emission lifetimes. These features are in agreement with the presence of two alkyl substituents on the diazine ligand (see Table 1), responsible for the shift at higher energy of the $^3\text{MLCT}$ excited state. The subsequent increase of the emission quantum yield and of the lifetime is in agreement with energy gap law.¹⁶

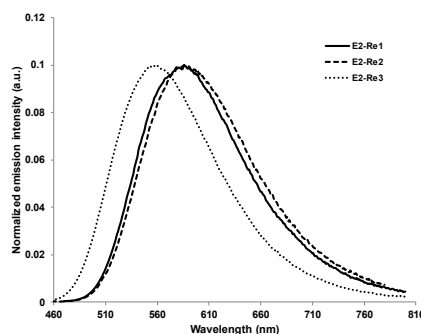


Fig. 1: Normalized emission spectra of Re-estradiol conjugates in de-aerated toluene solution at room temperature

Cell cultures

After chemical characterization, we moved to investigate the behavior (cell internalization and bio-imaging properties) of the three conjugates **E2-Re1**, **E2-Re2** and **E2-Re3** in biological systems, namely, simple cell cultures system and complex multicellular organisms. As for the former, we performed cellular uptake experiments of the different compounds on human breast carcinoma cell line MCF7. These cells are known to be estrogen-sensitive since express high levels of E2 receptors.¹³ In general, none of the tested compound resulted cytotoxic: indeed, at all the tested concentrations/exposure times cells were firmly attached to the substrate and their morphology was comparable to the controls (Fig. 2-4, Fig. S2), both these features being indications of their actual viability. As depicted in Fig. 2-4, we showed that only the conjugate **E2-Re1** was clearly internalized by living MCF7 cells. In order to understand the effect of initial concentration of staining complex on the cellular behavior at a specific incubation time, we carried out concentration dependent uptake experiments. Fig. 2 shows that conjugate **E2-Re1** was uptake when concentrations were higher than 30 μM and incubation times were at least 60 min. After this threshold, accumulation of the marker was rather similar in the different experimental conditions.

On the contrary, conjugates **E2-Re2** and **E2-Re3** displayed the general tendency to form aggregates in the culture medium, hampering their internalization in the cell. Only at the highest concentration (50 μM) and for short-incubation times (30 min) a partial up-take was observed for both compounds (see Fig. 3 and 4). This marked difference in cellular behavior, between the three different conjugates, could be related to the nature of the spacer linking the organometallic fragment to the C(17) of the estradiol moiety. In particular, these data could be interpreted in terms of (a) the hydrophobic versus hydrophilic character of the diazine substituents; (b) the steric bulk and the conformational flexibility of the 17 chain. Indeed, in **E2-Re3** the presence of the long alkyl chain allows the organometallic fragment to rotate freely around the estradiol moiety, sweeping out a great cone angle and increasing the steric hindrance then reducing the cellular uptake.

Moreover the presence of the methyl substituent in the β position of the diazine ligand of **E2-Re3**, while increases the lipophilicity of the conjugate, reduces the solubility in the cellular medium, affording the precipitation of the complex before the cellular up-take occurs.

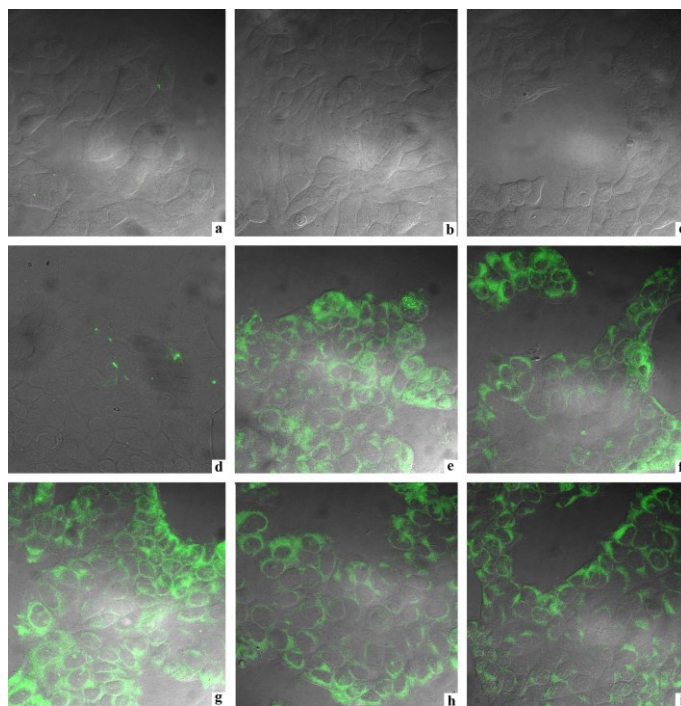


Fig. 2 Merged confocal images (bright field + green fluorescence) of MCF-7cell after addition of the compound **E2-Re1** at different concentrations (50 μM , 30 μM and 10 μM in 1% DMSO containing serum-free EMEM) and different incubation times (30, 60, 120 min). Accumulation inside the cells is clearly visible for concentrations higher than 30 μM and incubation time longer than 60 min. a: 10 μM , 30 min; b: 10 μM , 60 min; c: 10 μM , 120 min; d: 30 μM , 30 min; e: 30 μM , 60 min; f: 30 μM , 120 min; g: 50 μM , 30 min; h: 50 μM , 60 min; i: 50 μM , 120 min;

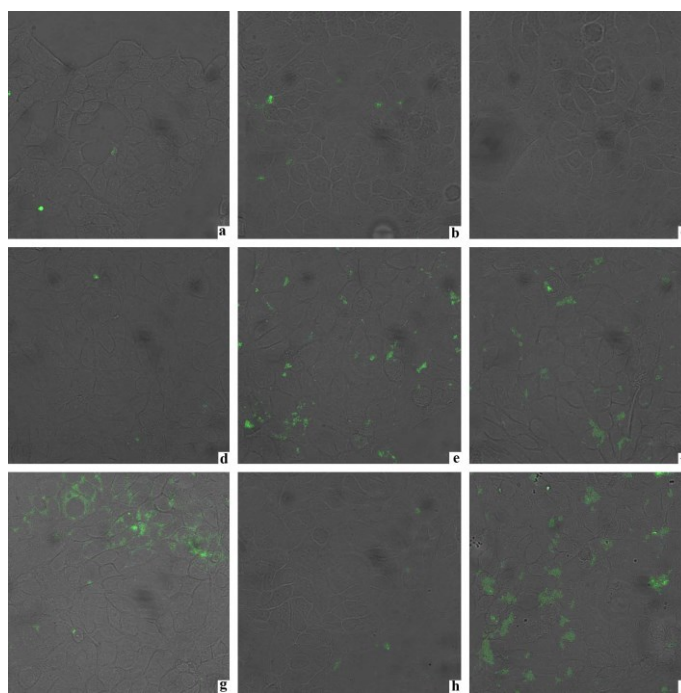
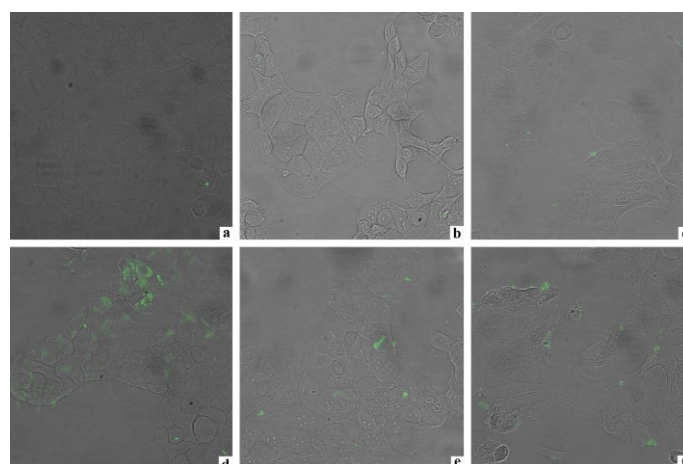


Fig. 3 Merged confocal image (bright field + green fluorescence) of MCF-7cell after addition of the compound **E2-Re2** at different concentrations (50 μM , 30 μM and 10 μM in 1% DMSO containing serum-free EMEM) and different incubation times (30, 60, 120 min). A scarce internalization of the compound is generally observed (aggregates are visible outside the cells) except for the 50 μM , 30 min condition where a partial accumulation is visible within the cells. a: 10 μM , 30 min; b: 10 μM , 60 min; c: 10 μM , 120 min; d: 30 μM , 30 min; e: 30 μM , 60 min; f: 30 μM , 120 min; g: 50 μM , 30 min; h: 50 μM , 60 min; i: 50 μM , 120 min;



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Fig. 4 Merged confocal images (bright field + green fluorescence) of MCF-7 cell after addition of the compound **E2-Re3** at different concentrations (50 μM , 30 μM in 1% DMSO containing serum-free EMEM) and different incubation times (30, 60, 120 min). No internalization is observed for concentration of 30 μM and at 50 μM only partial up-take is visible at short incubation time (30 min) whereas mainly extracellular aggregates can be observed after 60 and 120 min of incubation. a: 30 μM , 30 min; b: 30 μM , 60 min; c: 30 μM , 120 min; d: 50 μM , 30 min; e: 50 μM , 60 min; f: 50 μM , 120 min;

Multicellular organism (*C. intestinalis* embryos)

Experiments with MCF7 cells allowed selecting the proper compound (**E2-Re1**) to be tested in multicellular organisms such as *C. intestinalis* embryos. In these organisms, **E2-Re1** cellular uptake has been observed at both tested developmental stages. In 76-cell stage embryos (Fig. 5), the compound penetrated the cellular membrane of the outer cells, staining their cytoplasm and giving rise to intracellular emission centered in the green portion of the visible spectrum, with a slight extent in the red one (Fig. 5B and 5D). No intracellular emission light has been observed in the blue region of the visible spectrum (Fig. 5C), thus confirming that the cellular emission behavior is very similar to that previously observed from photophysical measurements in solution. At late tailbud stage, **E2-Re1** was slightly distributed in all the embryos. Higher concentrations have been detected in the dorsal ectoderm of both trunk and tail, at the level of the developing palps and in a trunk mesenchymal region. As in the previous stage, the fluorescence coming from the organometallic probe was detectable in both green (Fig. 6B) and red (Fig. 6D) channels but not in the blue one (Fig. 6C).

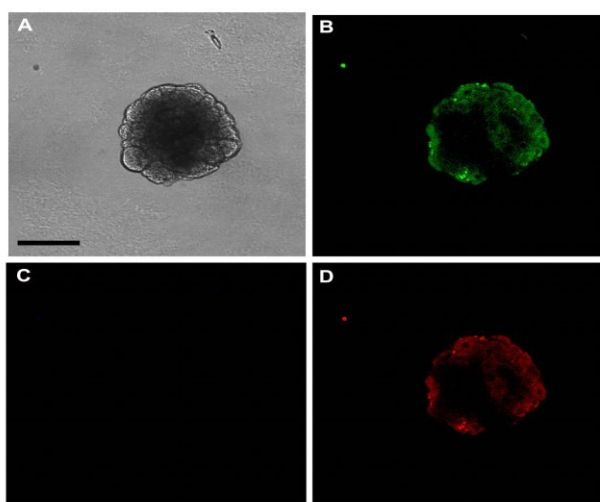


Fig. 5 *C. intestinalis* embryo at 76-cell stage exposed to **E2-Re1** for 3 h. A) Bright field; B) green fluorescence; C) blue fluorescence; D) red fluorescence. Scale bar = 50 μm .

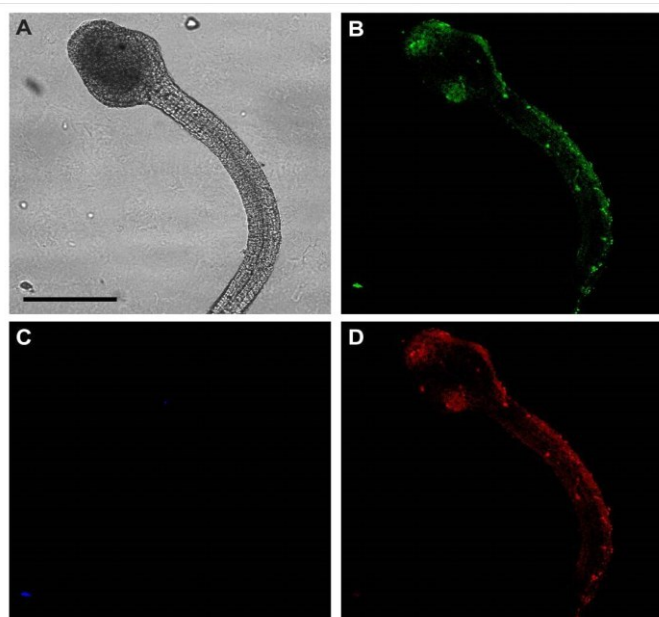


Fig. 6 *C. intestinalis* embryo at late tailbud stage exposed to **E2-Re1** for 3 h. A) Bright field; B) green fluorescence; C) blue fluorescence; D) red fluorescence. Scale bar = 50 μm .

Receptor targeting

To verify the possible interaction and specificity of the **E2-Re1** conjugate (the only conjugate able to efficiently stain the cells) with the estradiol receptor targeted “negative control” experiments have been performed. In order to evaluate if the presence of the estradiol moiety could mediate the cellular uptake and/or the localization inside the cell, in parallel to **E2-Re1** conjugate (Fig. 7a), we therefore investigated the behavior of estradiol-free rhenium complex $[\text{Re}_2(\mu\text{-Cl})_2(\text{CO})_6(\mu\text{-4Me-pydz})]$ (**Re-4Me**) (Fig. 7b) or the effect of an estradiol excess on **E2-Re1** up-take in MCF-7 cells (Fig. 7c). In both cases a clear cellular staining was still observable, thus suggesting a lack of specificity of the probe and indicating that, as already observed for the other Re-conjugates^{11,12} the mechanism of the up-take is mainly by passive diffusion and only partially, if not, mediated by the presence of the biological moiety and by specific interaction with ER. This feature is further confirmed by the up-take experiments performed on the 231 cell lines, which express low levels of ER. Figure S1 (see Supporting Information) shows that both the conjugate **E2-Re1** and the estradiol-free rhenium complex **Re-4Me** could stain these cells.

It is interesting to note that, in the case of MCF-7, **E2-Re1** was still uptake even in the presence of an excess of estradiol and the intensity of its emission was higher than in the absence of free estradiol (see Fig. 7c). This feature, in contrast to that usually observed in which the excess of estradiol displaces the probe from the binding with ER, suggests the occurrence of some quenching of the dye **E2-Re1** by the E2-receptor. Indeed, the observed quenching of the emission should be ascribed to the presence of some interaction, such as the hydrogen bond between the hydroxyl in position 3 of the estradiol unit of **E2-Re1** and some residues of the receptor.¹⁷

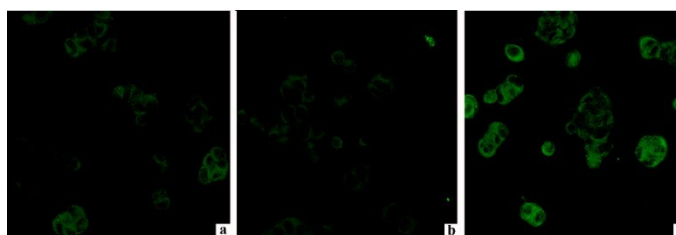


Fig. 7: Complex **E2-Re1** stains the positive MCF-7 cells: a) 50 μM of **E2-Re1** stains MCF-7 cells for 60 min. (B) 50 μM of fluorescent estradiol-free rhenium complex **Re-4Me** stains MCF-7 cells. (C) 50 μM of **E2-Re1** in the presence of 500 μM of estradiol stains the MCF-7 cells.

Conclusions

In summary three new fluorescent estrogen derivatives based on different dinuclear rhenium complexes have been successfully synthesized. The organometallic fragments have been covalently bound at the end of a rigid $\text{C}\equiv\text{C}$ ethynyl chain at the 17α position of the steroid using different synthetic strategies. These bio-conjugates have been evaluated in terms of their photophysical properties and suitability as imaging agent in living cells and in small multicellular organisms.

Although all the complexes were internalized by cells, even if in different amount, only **E2-Re1** provided a reliable bioimaging of cellular uptake. The marked differences among the compounds have been mainly ascribed to the nature of the spacer linking the organometallic fragment to C(17) of the estradiol, which modulates not only the photophysical properties, but also the interaction

between the probes and the cells. Unfortunately, the well-known ability of the dinuclear rhenium fragment to penetrate the cellular membranes, which in many cases allowed the cellular uptake of PNA oligomers, here hampers the possibility to exploit them for the detection of the estradiol receptor in the cells, thus confirming that parameters like solubility, size and lipophilicity of the probes must be controlled very well in order to obtain the best results.

On the other hand, these experiments have confirmed the previous findings concerning the good uptake and the low cytotoxicity of the dinuclear rhenium complexes, thus suggesting their possible future use as probes for imaging application in living cell. Therefore we can consider this work a preliminary study aimed to find the better probe, in a range of structural analogs and with the same photophysical properties, in terms of emission intensity, stability, cytotoxicity and cell uptake. Future work is planned to modify this molecular structure in order to increase the solubility in water and reduce the lipophilicity of the complex (and the use of DMSO in the cellular uptake) with the aim to make the internalization process more specific and selective. Experiments to better evaluate the binding with the estradiol receptor are also planned.

Finally, preliminary confocal emission imaging of the multicellular organism with the best performing dye **E2-Re1** have been also obtained. To the best of our knowledge, this is the first example of luminescent rhenium complex used to stain a multicellular organism, opening the way to the development of no-radioactive derivatives as efficient phosphorescent probes suitable for cell imaging.

Experimental

Materials and methods

Chemicals were purchased from commercial sources and used without further purification, unless otherwise indicated. When anhydrous conditions were required, the reactions were performed in oven-dried glassware under a nitrogen atmosphere. All the solvents were deoxygenated and dried using standard methods before use; toluene, Et₂O and dioxane were distilled on Na(s), while CH₂Cl₂ on P₂O₅. Commercial deuterated solvents were used as received. Column chromatography was performed using Alfa Aesar silica gel 60 (0.032–0.063 mm). ¹H NMR spectra were recorded on a Bruker DRX-400 MHz instrument, equipped with a Bruker 5 mm BBI Z-gradient probe head with a maxima gradient strength of 53.5 G cm⁻¹. IR spectra in solution were acquired on a Bruker Vector 22 FT spectrophotometer. Elemental analyses were performed on a Perkin Elmer CHN2400 instrument. ESI-MS spectra were acquired on LCQ Fleet ion trap mass spectrometer (Thermo Fisher). Electronic absorption spectra were recorded on an Agilent Model 8543 spectrophotometer at room temperature, and using quartz cells with a 1.0 cm path length.

Synthesis

Synthesis of the alkylated pyridazine ligands D1-D3. All the alkylated pyridazines were prepared according to a literature procedure,¹⁸ involving as first step the synthesis of 1,2,4,5-tetrazine (from hydrazine hydrate and formamide acetate) and then its reaction with 17 α -ethynylestradiol for **D1**, hex-5-ynoic acid for **D2** and undec-9-ynoic acid for **D3** pyridazine ligand. **D1** and **D2** were isolated as pure white solid by addition of Et₂O or *n*-hexane to a concentrated CH₂Cl₂ solution of **D1** and **D2**, respectively (isolated yield 47% for **D1** and 68% for **D2**). **D3** was not isolated as pure solid and was used in mixture with the corresponding alkyne. The purity of the ligands was checked by ¹H-NMR spectroscopy. **3-(17 α -estradiol)pyridazine (D1):** ¹H NMR (Acetone-d₆, 298 K): δ = 9.29 (1H, s, H_a pydz), 9.13 (1H, d, H_b pydz), 7.59 (1H, d, H_c pydz), 7.09 (1H, d, H₄), 6.54 (2H, m, H₁, H₂), 4.67 (1H, s, 3-OH), 2.81 (1H, m, H₆), 2.5–2.0 (3H, m, H_{11 α} , H_{11 β} , H_{12 β}), 2.00–1.6 (5H, m, H₉, H_{15 α} , H_{15 β} , H_{7 α} , H_{16 α}), 1.5–1.3 (4H, m, H₈, H₁₄, H_{7 β} , H_{12 α}), 1.15 (3H, CH₃), 0.5 (1H, m, H_{16 β}) ppm. **8-(5-methylpyridazin-4-yl)-octanoic acid (D3):** ¹H NMR (CD₂Cl₂, 298 K): δ = 8.89 (s, 2H, H_{ortho} accidental overlap), 2.65 (pt, 2H, CH₂-(CH₂)₅-CH₂-COOH), 2.40 (t, J = 7.0, 2H, CH₂-(CH₂)₅-CH₂-COOH), 2.36 (s, 3H, CH₃), 1.76–1.30 (m, 10H, CH₂-(CH₂)₅-CH₂-COOH) ppm.

Synthesis of the Complexes Re2, Re3 and Re-4Me. The complexes (see Schemes 3 for their structures and abbreviations) were prepared from [ReCl(CO)₅], using the method previously reported.^{10a, 11a, 19} The crude products were purified by addition of *n*-hexane to a concentrated solution in CH₂Cl₂, affording pure yellow powders in high isolated yields (40–80%). **[Re₂(μ -Cl)₂(CO)₆(μ -4-(pyridazin-4-yl)-butanoic acid)] (Re2)^{11a}: ¹H NMR (CD₂Cl₂, 400 MHz, 298 K): δ = 9.68 (2H, m, H_a+H_b pydz), 7.90 (1H, m, H_c pydz), 3.01 (2H, m, CH₂-CH₂-CH₂-COOH), 2.59 (2H, m, CH₂-CH₂-CH₂-COOH), 2.14 (2H, m, CH₂-CH₂-CH₂-COOH) ppm. FT-IR (CO) toluene: 2049 (m), 2033 (s), 1947 (s), 1914 cm⁻¹. Anal. calcd for C₁₄H₁₀Cl₂N₂O₈Re₂: C 21.63, H 1.30, N 3.60; found: C 21.40, H 1.41, N 3.56. **[Re₂(μ -Cl)₂(CO)₆(μ -8-(5-methylpyridazin-4-yl)-octanoic acid)] (Re3):** ¹H NMR (CD₂Cl₂, 400 MHz, 298 K): δ = 9.49 (1H, s, H_a), 9.45 (1H, s, H_b pydz), 2.86 (2H, m, CH₂-COOH), 2.58 (3H, s, CH₃), 2.39 (2H, m, pydz-CH₂), 1.8–1.4 (10H, CH₂ aliphatic chain). Anal. calcd for C₁₉H₂₀Cl₂N₂O₈Re₂: C 26.92, H 2.38, N 3.30; found: C 27.05, H 2.35, N 3.15.**

[Re₂(μ -Cl)₂(CO)₆(μ -4-methylpyridazine)] (Re-4Me)¹⁹: ¹H-NMR: δ = 2.68 (s, 3H, CH₃), 7.86 (dd, 1H), 9.64 (d, 1H, J = 5.8 Hz), 9.67 (d, J = 1.3 Hz, 1H). FT-IR ν (CO) = 2050 (m), 2034 (s), 1947 (s), 1915 cm⁻¹ (s). Anal. calcd for C₁₁H₆Cl₂N₂O₆Re₂: C 18.73, H 0.86, N 3.97; found: C 19.08, H 0.79, N 4.05.

Synthesis of the 4-(17 α -ethynylestradiol)aniline (E0). 17 α -ethynylestradiol (300 mg, 1.01 mmol) was added as a solid to a solution Pd(PPh₃)₂Cl₂ (28 mg, 4 mol %), and CuI (15.4 mg, 8 mol %) in 25 mL CH₂Cl₂ and 5 ml of triethylamine (TEA) under an inert atmosphere of nitrogen. The solution was stirred at room temperature for ten minutes. 4-iodo-anilina (221.6 mg, 1.012 mmol) was dissolved in 4

ml of CH₂Cl₂ under an inert atmosphere of nitrogen at room temperature and then slowly added to the solution containing the 17 α -ethynylestradiol. The color of the mixture turned to yellow after three hours and the mixture was then stirred for 24 h. The solid waste removed by filtration and, after the extraction with CH₂Cl₂, the organic phase was washed with small amount of NH₄Cl, brine and water and evaporated to dryness. The crude product was therefore purified by column chromatography on silica gel (CH₂Cl₂/AcOEt = 9/1) affording **E0** as pale yellow solid. Yield: 276 mg (70%). ¹H NMR (CD₂Cl₂, 400 MHz, 298 K): δ = 7.94 (1H, d, H1 estradiol), 7.14 (2H, d, H_b+H_{b'}), 6.62 (3H, m, H_a+H_{a'} and H2 estradiol), 6.54 (1H, H4 estradiol), 4.91 (1H, s, 3-OH), 4.27 (2H, NH₂), 2.77 (2H, m, H6), 2.4-2.15 (3H, m, H11 α , H9, H12 β), 2.10-1.70 (6H, m, H14, H15 α , H7 α , H16 β , H16 α , H11 β), 1.5-1.3 (3H, m, H8, H12 α , H7 β), 0.95 (3H, CH₃)

Synthesis of [Re₂(μ -Cl)₂(CO)₆(μ -3-(17 α -estradiol)pyridazine)] (E2-Re1). The complexes **E2-Re1** was prepared from [ReCl(CO)₅], using the method previously reported,¹⁰ in the presence of 0.5 equivalents of pyridazine ligand **D1**, in toluene solution. The crude product was purified by column chromatography on silica gel (AcOEt/*n*-hexane = 1/1), affording a pure yellow powder (19.3 mg, isolated yield 36%). ¹H NMR (CD₂Cl₂, 400 MHz, 298 K): δ = 9.90 (1H, s, H_a pydz), 9.72 (1H, d, H_b pydz), 7.87 (1H, d, H_c pydz), 7.09 (1H, d, H1), 6.57 (2H, m, H2, H4), 4.71 (1H, s, 3-OH), 2.86 (2H, m, H6), 2.55-2.2 (3H, m, H11 α , H11 β , H12 β), 2.10-1.75 (5H, m, H9, H15 α , H15 β , H7 α , H16 α), 1.6-1.3 (4H, m, H8, H14, H7 β , H12 α), 1.22 (3H, CH₃), 0.62 (1H, m, H16 β) ppm. FT-IR ν (CO) toluene: 2051 (m), 2034 (s), 1949 (s), 1917 cm⁻¹. ESI-MS: *m/z* 959.39 [M-2CO]⁺. Anal. calcd for C₃₀H₂₆Cl₂N₂O₈Re₂: C 34.96, H 2.72, N, 2.91; found: C 34.90, H 3.01, N 2.63.

Synthesis of [Re₂(μ -Cl)₂(CO)₆(μ -4-(pyridazin-4-yl)-butanoic-E0 amide)] (E2-Re2). A solution containing the complex **Re2** (20 mg, 0.0257 mmol), HATU (12.07 mg, 0.0308 mmol) and HOBt (4.28 mg, 0.0308 mmol) in 1 ml of CH₂Cl₂ and 5.38 μ L (0.0358 mmol) of TEA and 100 μ L di AcOEt was stirred at room temperature for 10 minutes. Then 19 mg (0.0514 mmol) of estradiol precursor **E0** and 7.17 μ L (0.0514 mmol) of TEA were added. The reaction was stirred at room temperature for 24 hours and therefore it was evaporated to dryness under reduced pressure to give a yellow solid. The crude product was purified by column chromatography on silica gel (CH₂Cl₂/ AcOEt = 7/3) affording 14.8 mg of isolated pure powder (50.2%). ¹H NMR (CD₂Cl₂, 400 MHz, 298 K): δ = 9.70-9.69 (2H, m, H_c+H_d pydz), 7.95 (1H, m, H_e pydz), 7.36 (1H, d, H1 estradiol), 7.26 (2H, dd, H_b+H_{b'}), 6.87 (1H, m, H2 estradiol), 6.83 (1H, m, H4 estradiol), 6.64 (2H, dd, H_a+H_{a'}), 3.90 (1H, broad, 3-OH), 3.07 (2H, m, CH₂-CH₂-CH₂-COOH), 2.90 (2H, m, H6), 2.77 (2H, m, CH₂-CH₂-COOH), 2.48-2.27 (3H, m, H11 α , H9, H12 β), 2.21 (2H, m, CH₂-CH₂-COOH), 2.15-1.75 (7H, m, H14, H15 α , H15 β , H7 α , H16 β , H16 α , H11 β), 1.5-1.35 (3H, m, H8, H12 α , H7 β), 0.95 (3H, CH₃) ppm. FT-IR ν (CO) CH₂Cl₂: 2047 (m), 2032 (s), 1948 (s), 1916 cm⁻¹. Positive ion ESI-MS: *m/z* = 1146.24 [M]⁺. Anal. calcd for C₄₀H₃₇Cl₂N₃O₉Re₂: C 41.88, H 3.25, N, 3.66; found: C 41.80, H 3.36, N 3.54.

Synthesis of [Re₂(μ -Cl)₂(CO)₆(μ -8-(5-methylpyridazin-4-yl)-octanoic-E0 amide)] (E2-Re3). A solution containing the complex **Re3** (30 mg, 0.0353 mmol), HATU (16.60 mg, 0.0423 mmol) and HOBt (5.89 mg, 0.0423 mmol) in 2 ml of CH₂Cl₂ and 7.44 μ L (0.0529 mmol) of TEA and 200 μ L di AcOEt was stirred at room temperature for 10 minutes. Then 27.42 mg (0.0707 mmol) of estradiol precursor **E0** and 9.93 μ L (0.0707 mmol) of TEA were added. The reaction was stirred at room temperature for 24 hours and therefore it was evaporated to dryness under reduced pressure to give a yellow solid. The crude product was purified by column chromatography on silica gel (CH₂Cl₂/ AcOEt = 7/3) affording 12.1 mg of isolated pure powder (28%). ¹H NMR (CD₂Cl₂, 400 MHz, 298 K): δ = 9.49 (1H, s, H_c pydz), 9.45 (1H, s, H_d pydz), 7.34 (1H, d, H1 estradiol), 7.26 (2H, dd, H_b+H_{b'}), 6.83 (1H, m, H2 estradiol), 6.79 (1H, m, H4 estradiol), 6.64 (2H, m, H_a+H_{a'}), 3.90 (1H, broad, 3-OH), 2.87 (4H, m, -CH₂-COOH and H6 estradiol), 2.59-2.57 (5H, m, CH₃ and CH₂ aliphatic chain), 2.50-2.25 (3H, m, H11 α , H9, H12 β), 2.17-1.90 (3H, m, H12 α , H16 α , H11 β), 1.85-1.67 (5H, m, H14, H15 α , H15 β , H16 β , H7 β), 1.6-1.20 (14H, m, H8, H7 α , and 10H of CH₂ aliphatic chain), 0.95 (3H, CH₃) ppm. FT-IR ν (CO) CH₂Cl₂: 2045 (m), 2034 (s), 1948 (s), 1915 cm⁻¹. ESI-MS: *m/z* 1215.60 [M]⁺. Anal. calcd for C₄₅H₄₇Cl₂N₃O₉Re₂: C 44.40, H 3.89, N 3.45; found: C 44.46, H 3.99, N 3.37.

Photophysical characterization

Photophysical measurements were carried out in air-equilibrated and de-aerated toluene solutions at room temperature. Electronic absorption spectra were recorded on an Agilent Model 8543 spectrophotometer at room temperature, using quartz cells with 1.0 cm path length. Steady-state emission spectra were recorded on an Edinburgh FLS980 spectrometer equipped with a 450 W ozone-free xenon arc lamp, double grating excitation and emission monochromators (2 \times 300 mm focal length) and a Hamamatsu R928P photomultiplier tube. Time-resolved measurements were performed using the time-correlated single-photon counting (TCSPC) option on the FLS980. The excitation sources were mounted directly on the sample chamber and the emission is collected by a multichannel plate MCP-PMT Hamamatsu H10720-01 single-photon-counting detector. The photons collected at the detector were correlated by a time-to-amplitude converter (TAC) to the excitation pulse. The data analysis was performed using the commercially available F980 software (Edinburgh Instruments). The goodness of the data fitting was assessed by minimizing the reduced chi-squared function (χ^2). Photoluminescence quantum yields (Φ) were collected on optically diluted solution ($<1.0 \times 10^5$ M) using wavelength scanning with a Hamamatsu C11347-11 Quantaurus-QY Absolute PL quantum yield spectrometer, equipped with a xenon light source (150 W), a monochromator, a Spectralon integrating sphere, and employing the commercially available U6039-05 PLQY measurement software (Hamamatsu Photonics Ltd, Shizuoka, Japan). The photoluminescence quantum yields were measured exciting the samples between 380–420 nm.

Cell cultures

MCF7 (human breast adenocarcinoma, cod. 86012803, Sigma Aldrich) cells were grown in basal growth medium as following: minimum essential medium Eagle (EMEM) with Earles salts and NaHCO₃, (Sigma) supplemented with 10% fetal calf serum (Euroclone), 2 mM glutamine (Euroclone), antibiotic antimycotic solution 100 U/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B (Sigma-Aldrich) at 37°C, 5% CO₂.

Cell culture testing

Cellular uptake of the three different complexes (**E2-Re1**, **E2-Re2**, **E2-Re3**) was investigated by using MCF7 breast cancer cells. Cells were seeded in 24x multiwells 4-5 days before the exposure experiments (cell density approximately 50000 cells/plate) and were cultivated (37°C, 5% CO₂) in basal growth medium (see Cell culture paragraph). Three different concentrations (10, 30, 50 µM) and time-points (30', 60', 120') were tested for each compound (in **E2-Re3** the 10 µM was omitted). For the receptor targeting (specificity) tests, in parallel to **E2-Re1** (50 µM, 30'), also estradiol-free rhenium complex [Re₂(µ-Cl)₂(CO)₆(µ-4Me-pydz)] (**Re-4Me**) and a mixture of **E2-Re1** (50 µM) and pure E2 (500 µM) were tested. All the Experiments were carried out in triplicates. The compounds were initially dissolved in DMSO and then added to serum-free EMEM (final DMSO concentration 1%). One mL of this solution was added to each well after removal of the serum-containing medium. At the end of the exposure period cells were washed with PBS and fixed with paraformaldehyde 4% for ten minutes. After further washings in PBS cells were mounted with Pro-long anti-fade reagent (P7481, Life Technologies) with DAPI to stain the nuclei. The images were acquired with a Leica TCS NT confocal microscope.

Cells were imaged by excitation at 405 nm and the resulting emissions were collected in three different spectral interval (485/30, 535/50, 600/40).

C. intestinalis testing

E2-Re1 uptake was investigated also in more complex biological systems i.e. multicellular organism (*Ciona intestinalis* embryos). Adults of *C. intestinalis* were collected by the fishing service of the Roscoff Biological Station (France). Animals were maintained in aquaria filled with artificial seawater (Instant Ocean; salinity about 32‰) and provided with circulation system as well as mechanical, chemical and biological filters. Constant light condition was preferred to promote gamete production. Gametes were obtained surgically from the gonoducts and dechorionated in filtered artificial seawater (ASW; pH 8.0) containing 1% sodium thioglycolate and 0.05% protease. *In vitro* cross-fertilization was performed and embryos were cultured in 1% agarose-coated petri dish in ASW at 18 °C up to the stages of interest. Embryos were exposed to 20 µM **E2-Re1** for 3 h: to obtain samples at 76-cell stage (4.2 hour post fertilization (hpf); Hotta et al., 2007) exposure period started at 4-cell stages (1.45 hpf) while late tailbud embryos (13 hpf) were exposed at mid tailbud stage (10 hpf). Embryos were then fixed with 4% Paraformaldehyde in PBS, mounted with 1,4-diazabicyclo[2,2,2]octane (DABCO, Sigma, Italy) on microscope slides and observed under a Leica TCS SP2 Laser Scanning Confocal microscope (Leica Microsystems) as above described.

Conflicts of interest

There are no conflicts to declare.

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