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# Remarkable Effects of a Rhenium(I)-diselenoether Drug on the Production of Cathepsins B and S by Macrophages and their Polarizations.

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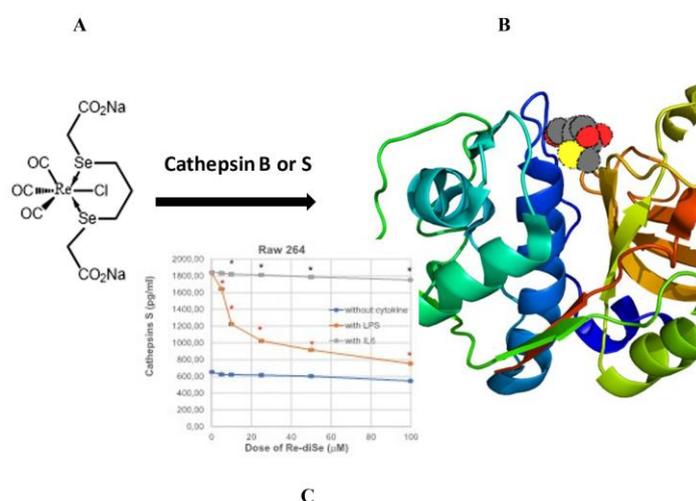
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**Running Title: Immune Interest of a Rhenium-diselenoether Compound**

**Graphical abstract:**



# Remarkable Effects of a Rhenium(I)-diselenoether Drug on the Production of Cathepsins B and S by Macrophages and their Polarizations.

## Abstract:

Background/Objective. Tumor-associated macrophages (TAMs) produce an excessive amount of cysteine proteases, and we aimed to study the effects of anticancer rhenium(I)-diselenoether (Re-diSe) on the production of cathepsins B and S by macrophages. We investigated the effect of Re-diSe on lipopolysaccharides (LPS) induced M1 macrophages, or by interleukin 6 (IL-6) induced M2 macrophages. Methods. Non-stimulated or prestimulated murine Raw 264 or human THP-1 macrophages were exposed to increasing concentrations of the drug (5, 10, 20, 50 and 100  $\mu$ M) and viability was assayed by MTT assay. The amount of cysteine proteases was evaluated by ELISA tests, the number of M1 and M2 macrophages by the expression of CD80 or CD206 biomarkers. The binding of Re-diSe with GSH as a model thiol containing protein was studied by mass spectrometry. Results. A dose-dependent decrease of cathepsins B and S was observed in M1 macrophages. There was no effect in non-stimulated cells. The drug induced a dramatic dose-dependent increase in M1 expression in both cells, significantly decreased the M2 expression in Raw 264 and had no effect in non-stimulated macrophages. The binding of the Re atom with the thiols was clearly demonstrated. Discussion. The increase in the number of M1 and a decrease in M2 macrophages treated by Re-diSe could be related to the decrease in cysteine proteases upon binding of their thiol residues with the Re atom.

**Key-words:** Rhenium (Re), rhenium(I)-diselenoether (Re-diSe), macrophages, immune resistance, cysteine proteases, cancer

## 1. INTRODUCTION

The rhenium(I)-diselenoether (Re-diSe) is a metal-based anticancer drug under preclinical development [1]. This complex features a central tricarbonyl fac-[Re(CO)<sub>3</sub>] core coordinated by a diselenide ligand (two Se atoms). It is simply obtained by ligand exchange reaction of pentacarbonylchlororhenium (I) with 3,7-diselena nonanedioic acid and sodium salt formation [2].

Recent publications related the potential interest of Re-based drugs to treat resistant cancers, with a high selectivity towards cancer cells [3–12]. Among them, the Re-diSe drug has the advantage of having been extensively studied. It demonstrated its selectivity against cancer cells [13], even when the medium of culture of the cells was enriched in inflammatory cytokines [14], but also its antitumor activity in tumour-bearing animals [15,16], pharmacological [17,18] and toxicological data [19]. However, controversial results have also been

obtained, with pro-tumor effects, in some experimental models [19,20]. They could be explained by the mechanism of action of Re-diSe on the oxidative stress as previously described [19]. However, the lack of effect of Re-diSe in tumour-bearing mice pretreated by a total body irradiation suggested it may interfere with immune cells.

Moreover, a study showed a significant decrease of cathepsins cysteine proteases B and S upon treatment with Re-diSe in both cancer and normal cells and confirmed the selective inhibitory effects on MDA-MB231 breast cancer lines by comparison with normal HEK-273 embryonic kidney cells [14,21]. Cysteine proteases B, L and S, which are produced in excess in cancer cells, promote cancer growth and neovascularization and are key regulators of the innate and adaptive arms of the immune system [22,23]. They are produced in a high quantity in immune cells of the tumor micro-environment (TME), such as tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells [24]. We thus aimed to investigate the effects of the Re-diSe drug on macrophages. TAMs affect tumor growth, tumor angiogenesis, immune regulation, metastasis, but more importantly are involved in the resistance of cancer cells [25,26]. The accumulation of macrophages in TME is largely associated with poor disease out-come [27]. More particularly, TAMs tightly regulate tumor metastasis in all of the steps involved [28]. However, all macrophages do not have the same functions [29]. The functional M0 polarization represents the naïve macrophage status (uncommitted macrophages), which can be activated as classically activated M1 macrophages (TH1-driven), or as alternatively activated M2 macrophages (TH2-driven) [25]. In cytokine-deficient medium, the polarized macrophages revert back to the M0 state [30].

M1 macrophages stimulate the inflammation response against tumor cells. They produce pro-inflammatory cytokines (IL-1 $\beta$ , TNF, IL-6, IL-12, IL-23) and NO [31, 32]. They have a cytotoxic effect on transformed cells [28], and classical macrophage activation is crucial for killing cancer cells. The M1 polarization can be induced by lipopolysaccharide (LPS) either alone or in association with Th1 cytokines such as IFN- $\gamma$ , GM-CSF, interferon-gamma (IFN $\gamma$ ) or tumor necrosis factor-alpha (TNF- $\alpha$ ).

On the other hand, M2 macrophages are known to exert an immune suppressive phenotype, favoring tumor resistance. M2 macrophages represent the prominent population in the TME. They express anti-inflammatory cytokines, such as interleukin-10 (IL10), and tumor growth factor- $\beta$  (TGF- $\beta$ ) [32]. They may more precisely be classified into four subdivisions: alternative activated macrophages (M2a, activated by IL-4 or IL-13), type 2 macrophages (M2b, activated by immune complexes and LPS), deactivated macrophages (M2c, activated by glucocorticoids or IL-10), and M2-like macrophages (M2d, activated by adenosines or IL-6) [33]. Alternatively

activated or M2 macrophages, which are anti-inflammatory and immunoregulatory are polarized by Th2 cytokines such as IL-4 and IL-13. It was also identified that IL-6, mainly produced by endothelial cells and fibroblasts, may promote the alternative macrophage activation in TME [34], but also in infectious diseases, regulating the excessive release of NO through IL-1 $\beta$  inhibition [31]. In a clinical trial assay in metastatic triple-negative breast cancer patients, increased levels of IL-6 with greater expression of markers associated with M2 macrophage polarization were observed in resistant patients to a chemotherapy with taxane and an inhibitor of Nitric Oxide Synthase (NOS) [35]. However, IL-6 is also a pro-inflammatory cytokine, produced in endothelial cells and fibroblasts [36], and a marker of inflammation [37,38].

A switch is possible from M2 to M1 thanks to the plasticity of these macrophages, by changes in the characteristics of the TME [39]. The inhibition of cathepsins B, S and L may induce a re-polarization from M2 towards an M1-like phenotype with an increased expression of several typical M1 mediators, including IL-1, IL-6, CCL2, TNFA, NOS2, NFkBp65, CCR7, and FASN in the repolarized macrophages [40].

It was one of our objectives to look for changes in M1/M2 states under the influence of the Re-diSe drug. As these changes were expected *via* a decreased production of cysteine proteases B and S by the macrophages, we evaluated by ELISA tests their secretion in the medium of culture after treatment by Re-diSe.

Two types of cells were used in this experiment to mimic M1 and M2 macrophages, mouse Raw-264 and human THP-1. Human THP-1 cells is a human leukemia monocytic cell line suitable for studying immunomodulating new drugs [41,42] and the molecular characterization of four M2 subtypes (M2a, M2b, M2c, and M2d) has even been defined in these cells by a proteomic assay with very similar protein expression profiles [43]. A review of 85 articles highlighted the relevance of using THP-1-derived macrophage as a useful alternative to primary macrophage to study new anticancer agents on the polarization of macrophages [44]. Raw 264.7 is a mouse leukemia cell line of monocyte macrophage. The protein expression profiles of M1 and M2 phenotypes from both human THP-1 and mouse RAW264.7 macrophages were systematically investigated using mass spectrometry-based proteomics revealing their divergences [45]. The authors identified a list of proteins that were uniquely up-regulated in each macrophage type. Raw 264.7 cells could be more specifically used to investigate the lipopolysaccharide (LPS)-induced inflammatory markers, including nitric oxide (NO) [46].

We determined the effects of different doses of Re-diSe (5, 10, 20, 50 and 100  $\mu$ M), for an exposure time of 120h, on the viability of macrophages. We studied then the effects of the different doses of the drug at this exposure time on the production of cathepsins cysteine proteases B and S after stimulation or not of the macrophages. We chose LPS for our study to stimulate the M0 to M1 macrophages, according to our previous

publications [14], and IL-6 to stimulate macrophages into M2 polarization. M1-like macrophages were assayed by the markers CD80 [30] and M2 macrophages by the mannose receptor CD206 [34].

LPSs induce nitric oxide (NO) [47], and also iNOS-2 [48]. Plasma NO concentrations were increased in broilers after intravenous injections of LPS [49]. Due to the main effect of LPS in the NO production, we also assayed the effects of Re-diSe on this marker in M1-like macrophages.

Finally, we investigated the binding of Re-diSe with N-acetylcysteine and glutathione, used as a simple model of thiol (SH) containing peptide to look for a possible explanation of the decreased expression of cysteine proteases by Re-diSe, as it was already shown that Re complexes could bind to the SH group of cysteine proteases and inhibit their activity [50,51].

## **2. MATERIAL AND METHODS**

### **2.1 Cell Lines**

Cell lines (Raw 264 and THP-1 cells) were purchased from National Centre for Cell Science (NCCS), Pune, India and maintained in the CO<sub>2</sub> incubator as per the standard protocol. Dimethyl sulfoxide (DMSO), fetal bovine serum (FBS), Rosewell park memorial institute (RPMI), penicillin, streptomycin, amphotericin B, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), ethidium bromide (EB) were purchased from Sigma Aldrich, USA. ELISA kits for the biomarkers were obtained by MyBioSource Inc, USA. The rhenium complex was synthesized as previously described [13] and its structure is depicted as supplementary material (S1). N-acetyl cysteine and glutathione were purchased from Sigma-Aldrich (France).

The cell lines (Raw 264 and THP-1 cells) at cell density of  $1 \times 10^6$  cells/ml were seeded in 96 well plates for 12 hours. Cells were prior treated or not by LPS (1ng/mL) or IL6 (10ng/mL) for 24h and then exposed to the different doses (5, 10, 20, 50 and 100  $\mu$ M) of the Re-diSe drug for 120h. The medium of culture consisted of DMEM supplemented with 10% FBS, 10 U/ml of penicillin, 10 mg/ml of streptomycin, and 0.25 mg/ml of amphotericin-B maintained in a CO<sub>2</sub> incubator at 37°C and 5% CO<sub>2</sub>.

### **2.2. MTT Assay**

Assays were performed in non-stimulated cells after exposure to the drug with a comparison with non-treated cells. After incubation, the cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent for 3 h. After 3 h, the medium was removed and formazan crystals produced were dissolved in dimethyl sulfoxide. The absorbance was read at 600 nm and the relative absorbance was recorded, using multiwell plate reader.

### **2.3. Biomarkers**

Membrane protein extraction was performed with cells obtained after exposure to experimental condition using Mem-PERTM Plus membrane protein extraction kit (89842), Thermo Fischer Scientific, India. For estimation of CD80 and CD206 levels, CD-80 ELISA kit (MBS705535) and CD 206 ELISA kit (MBS260241) was performed as per manufacturer's instructions.

#### **2.4. NO Estimation**

Griess Reagent was used to estimate the NO produced in the medium upon incubation using sodium nitrite as standard.

#### **2.5. Assays of Cathepsins**

The supernatant was collected and stored at  $-80^{\circ}\text{C}$  after the end of the experiment until use. Cathepsins B and S were assayed by ELISA tests in the culture medium using ELISA kits from ELabsience (Houston, TX, USA).

#### **2.6. Binding of Re-diSe with the Cysteine of N-Acetylcysteine and Glutathione (GSH)**

A solution of the rhenium complex (6.9 mg, 0.01 mmol) and N-acetylcysteine (1.7 mg, 0.01 mmol) in water (0.6 mL) was incubated at  $37^{\circ}\text{C}$  for 48 h. After freeze drying the crude mixture was analyzed by mass spectrometry using a LTQ-Orbitrap Velos Pro (ThermoFisher) spectrometer using negative ion electrospray technic (ESI). The same method was used with 6.9 mg of rhenium complex (0.01 mmol) and 6.2 mg of GSH (0.02 mmol).

#### **2.7. Statistical analysis**

Results were expressed as mean  $\pm$ SD of triplicate experiments. The statistical analysis was performed using the SPSS software (IBM, Chicago, IL, USA). The comparison of the effects at different doses of the Re-diSe drug was analyzed using the Mann-Whitney U-tests. Results were considered statistically significant at  $p < 0.05$ .

### **3. RESULTS**

#### **3.1. Cell Viability**

The cell viability was determined in non-stimulated macrophages. There was a dose-dependent decreased of the % of viable cells up to about 20% at the highest dose of  $100\ \mu\text{M}$  Re-diSe. Results were significant at  $5\ \mu\text{M}$  ( $p < 0.05$ ) for Raw264 cells and at  $10\ \mu\text{M}$  for THP-1 cells. Each further dose significantly decreased the viability by comparison with the nearest lower value. At the dose of  $25\ \mu\text{M}$  Re-diSe, the cell viability was decreased by about 10% in both Raw264 and THP-1 cells. The effects are expressed in Fig. (1).

#### **3.2. Expression of Cathepsins Cysteine Proteases B and S**

##### **3.2.1. Expression of Cathepsins B**

Results are expressed in Fig. (2).

#### 3.2.1.1. In Non-treated Cells

LPS and IL-6 induced a highly and similar significant increase in the levels of cathepsins B, in both cells.

In Raw 264 cells, the basal levels of cathepsins B without any stimulation and without any treatment were  $525.20 \pm 0.82$  pg/mL. After stimulation by LPS they highly significantly increased up to  $1224.20 \pm 0.84$  pg/mL and at  $1112.20 \pm 0.84$  pg/mL after stimulation by IL-6.

In THP-1 cells, the basal levels of cathepsins B without any stimulation and without any treatment were  $418 \pm 15$  pg/mL. After stimulation by LPS they highly increased up to  $1219.15 \pm 0.59$  pg/mL and at  $1125.50 \pm 0.79$  pg/mL after stimulation by IL-6.

#### 3.2.1.2. In Cells Treated by Re-diSe

The Re-diSe treatments had no significant effect on non-stimulated macrophages in both cells. In macrophages stimulated by LPS, the Re-diSe drug induced a dramatic and highly significant dose-dependent decrease in the levels of cathepsins B in both cell lines.

In macrophages stimulated by IL-6, a significant decrease of cathepsins B was also observed, but more slightly, at doses of 5, 10 and 25  $\mu$ M, and did not further decrease at doses of 50 and 100  $\mu$ M Re-diSe:

- In Raw 264 macrophages these levels significantly decreased from  $1224.20 \pm 0.84$  pg/mL to  $1102.50 \pm 0.92$  pg/mL at the dose of 5  $\mu$ M Re-diSe, to  $1027.90 \pm 0.74$  pg/mL at the dose of 10  $\mu$ M Re-diSe and to  $989.70 \pm 0.53$  pg/mL at the dose of 25  $\mu$ M Re-diSe.
- In THP-1, the levels decreased from  $1125.50 \pm 0.79$  pg/mL to  $1110.60 \pm 0.54$  pg/mL at the dose of 5  $\mu$ M Re-diSe, to  $1092.70 \pm 0.76$  pg/mL at the dose of 10  $\mu$ M Re-diSe and to  $968.60 \pm 0.85$  pg/mL at the dose of 25  $\mu$ M Re-diSe.

### 3.3. Expression of Cathepsins S

Results are expressed in Fig. (3).

#### 3.3.1. In Non-treated Cells

Both LPS and IL-6 increased significantly and similarly the levels of cathepsins S.

In Raw 264 cells, the basal levels of cathepsins S without any stimulation and without any treatment were  $652.70 \pm 0.57$  pg/mL. After stimulation by LPS they significantly increased up to  $1835.80 \pm 0.92$  pg/mL and to  $1843.80 \pm 0.75$  pg/mL after stimulation by IL-6.

In THP-1 cells, the basal levels of cathepsins S without any stimulation and without any treatment were  $678.40 \pm 0.82$  pg/mL. After stimulation by LPS they significantly increased up to  $1923.50 \pm 0.89$  pg/mL and to  $1821.80 \pm 0.84$  pg/mL after stimulation by IL-6.

### 3.3.2. *In Cells Treated by Re-diSe*

The Re-diSe treatments had no significant effect on non-stimulated macrophages.

In Raw 264 and THP-1 macrophages stimulated by LPS, the Re-diSe drug induced a significant decrease in the levels of cathepsins S at doses of 5  $\mu$ M Re-diSe, and each further dose significantly continued to decrease these levels, as a high dose-dependent effect.

Effects in macrophages stimulated by IL-6:

- In Raw 264 macrophages, the Re-diSe drug significantly decreased the levels of cathepsins S from  $1843.80 \pm 0.75$  pg/ml to  $1832.70 \pm 0.78$  pg/mL at the dose of 5  $\mu$ M Re-diSe,  $1820.70 \pm 0.7$  pg/mL at the dose of 10  $\mu$ M Re-diSe,  $1811.50 \pm 0.84$  pg/mL at the dose of 25  $\mu$ M Re-diSe,  $1786.40 \pm 0.74$  pg/mL at the dose of 50  $\mu$ M Re-diSe,  $1752.60 \pm 0.65$  pg/mL at the dose of 100  $\mu$ M Re-diSe. Each dose decreased significantly the level by comparison with the nearest lower value.
- In THP-1 macrophages, the Re-diSe drug decreased the levels of cathepsin S from  $1821.80 \pm 0.92$  pg/mL to  $1823.50 \pm 0.83$  pg/mL at the dose of 5  $\mu$ M Re-diSe, to  $1802.80 \pm 0.92$  pg/mL at the dose of 10  $\mu$ M Re-diSe,  $1795.50 \pm 0.75$  pg/mL at the dose of 25  $\mu$ M Re-diSe,  $1728.50 \pm 0.58$  pg/mL at the dose of 50  $\mu$ M Re-diSe,  $1705.70 \pm 0.73$  pg/mL at the dose of 100  $\mu$ M Re-diSe. Only doses of 50  $\mu$ M Re-diSe were significant by comparison with non-treated cells.

## 3.4. Polarization of Macrophages

### 3.4.1. CD80 Markers of type M1 Macrophages (Polarization by LPS)

LPS induced a high significant increase of CD80 markers by comparison with non-stimulated macrophages, from  $0.14 \pm 0.12$  pg/mL to  $1.26 \pm 0.02$  pg/mL in Raw 264 macrophages and from  $0.15 \pm 0.01$  pg/mL to  $1.32 \pm 0.03$  pg/mL in THP-1 cells.

The Re-diSe drug induced a significant dose-effect increase in the quantity of macrophages de type M1 as evaluated by CD80 markers in cells stimulated by LPS. The increase of CD80 markers was significant at the dose of 5  $\mu$ M for Raw264 cells and at the dose of 10  $\mu$ M for THP-1 cells. No effect was observed on the non-stimulated macrophages (in non-inflammatory situations).

Results are expressed in Fig. (4).

#### 3.4.2. CD206 Markers of type M2 Macrophages (Polarization by IL-6)

IL-6 induced a high significant increase of CD 206 markers by comparison with non-stimulated macrophages, from  $2.62 \pm 0.04$  pg/mL to  $13.29 \pm 0.09$  pg/mL in Raw 264 macrophages and from  $2.47 \pm 0.07$  pg/mL to  $12.45 \pm 0.05$  pg/mL in THP-1 macrophages.

The Re-diSe drug had no effect in non-stimulated cells, in both types of cells.

In Raw cells stimulated by IL-6, there was a significant decrease in CD 206 markers, from  $13.29 \pm 0.09$  pg/mL to  $12.48 \pm 0.08$  pg/mL at the dose of 5  $\mu$ M Re-diSe. Further doses were not much effective, except the dose of 100  $\mu$ M with a significant decrease to  $10.95 \pm 0.11$  pg/mL by comparison with the dose of 50  $\mu$ M ( $11.85 \pm 0.05$  pg/mL).

In THP-1 cells stimulated by IL-6, the Re-diSe drug had no effect.

Results are expressed in Fig.(5).

### 3.5. Production of NO

Results on macrophages stimulated by LPS were obtained and expressed in Fig. (6).

#### 3.5.1. In Non-treated Cells

LPS induced a highly significant increase in NO quantity in macrophages by comparison with non-stimulated macrophages, from  $4.11 \pm 0.02$  to  $16.28 \pm 0.5$   $\mu$ M in Raw 264 cells and from  $3.65 \pm 0.01$  to  $22.58 \pm 0.7$   $\mu$ M in THP-1 cells.

#### 3.5.2. Treated Cells:

- In Raw 264 cells:
  - In non-stimulated cells by LPS, there was a significant increase in the NO quantity, from  $4.11 \pm 0.02$  to  $5.54 \pm 0.01$   $\mu$ M at the dose of 5  $\mu$ M Re-diSe. The levels increased but not significantly with further doses, to  $5.89 \pm 0.01$   $\mu$ M at the dose of 10  $\mu$ M Re-diSe,  $6.35 \pm 0.01$   $\mu$ M at the dose of 25  $\mu$ M Re-diSe,  $6.45 \pm 0.02$   $\mu$ M at the dose of 100  $\mu$ M Re-diSe.

- In cells stimulated by LPS, there was a non-significant decrease in the NO quantity, from  $16.28 \pm 0.5$  to  $16.15 \pm 0.3$   $\mu\text{M}$  at the dose of 5  $\mu\text{M}$  Re-diSe,  $15.96 \pm 0.8$   $\mu\text{M}$  at the dose of 10  $\mu\text{M}$  Re-diSe. The levels became significantly decreased to  $14.26 \pm 0.6$   $\mu\text{M}$  at the dose of 25  $\mu\text{M}$  Re-diSe, without significant difference at further doses ( $14.08 \pm 0.5$   $\mu\text{M}$  at the dose of 50  $\mu\text{M}$  Re-diSe,  $13.89 \pm 0.8$   $\mu\text{M}$  at the dose of 100  $\mu\text{M}$  Re-diSe).
- In THP-1 cells:
  - In non-stimulated macrophages, there was an increase in NO quantity by Re-diSe, from  $3.65 \pm 0.01$  to  $3.95 \pm 0.02$   $\mu\text{M}$  at the dose of 5  $\mu\text{M}$  Re-diSe,  $4.26 \pm 0.02$   $\mu\text{M}$  at the dose of 10  $\mu\text{M}$  Re-diSe,  $5.35 \pm 0.09$   $\mu\text{M}$  at the dose of 25  $\mu\text{M}$  Re-diSe,  $5.98 \pm 0.04$   $\mu\text{M}$  at the dose of 50  $\mu\text{M}$  Re-diSe. However, the levels became significantly increased only at the dose of 100  $\mu\text{M}$  Re-diSe ( $6.66 \pm 0.05$   $\mu\text{M}$ ).
  - In macrophages stimulated by LPS, there was no significant effect of Re-diSe in NO quantity.

### 3.6. Binding of Re-diSe to Cysteine of *N*-acetyl-cysteine and Glutathione

Analysis of the mass spectrum of the crude mixture from the reaction of *N*-acetyl cysteine with the rhenium complex showed beside the signal of the starting material ( $m/z = 586.9$ ) several sets of new signals. A first set of pick 315-319 was attributed to the free diselenide ligand displaying the expected isotopic pattern. A 1:1  $\text{Re}(\text{CO})_3$  cysteine adduct with  $m/z$  432.1 was observed together with two adducts containing two  $\text{Re}(\text{CO})_3$  and two and three *N*-acetyl cysteine residue at  $m/z$  862.92 and 1025.60 respectively. The formulas, molecular masses, potential structures, the observed and calculated isotopic patterns are depicted in the Table I.

Treatment of the rhenium complex with glutathione (GSH) (2 equiv.) provided minute amounts of the 1:1  $(\text{CO})_3\text{Re}/\text{GSH}$  ( $m/z$  576.1). The main product was a cluster with two  $\text{Re}(\text{CO})_3$  and three GSH similar to the one observed with *N*-acetyl cysteine but bis-charged ( $m/z = 729.6$ ) (Table 2).

The presumed mechanism of the reaction of Re-diSe with *N*-acetyl cysteine and GSH and the putative structure of the observed ions are described in supplementary material (S2 and S3).

## 4. DISCUSSION

### 4.1. Cell Viability

The viability of macrophages was affected by the Re-diSe treatment, but did not exceed 20% and only at the highest dose. Therefore, the results obtained for the biomarkers should not reflect a consequence of the inhibition of the cell growth, but an effect on the production of the studied markers by Re-diSe. In a previous study, with the same design in MDA-MB231 breast cancer cells in culture, with an exposure time of 120h, it was already

observed that a dose of 25  $\mu$ M of the Re-diSe drug inhibited more than 60% of the malignant cells, but less than 20% of the normal cells [13].

#### **4.2. LPS and IL-6, Distinct Inducers of Polarization of the Macrophages**

We showed that stimulation by LPS increased levels of cathepsins and expression of CD80 markers while stimulation by IL-6 increased expression of cathepsins and CD206, by comparison with non-stimulated macrophages. It was thus a suitable model to investigate the effects of Re-diSe after stimulation of the macrophages by these cytokines.

#### **4.3. Cathepsins**

The Re-diSe drug had no effect when the macrophages were not stimulated and this drug should be safe in non-inflammatory conditions. In contrast, Re-diSe significantly decreased the levels of the two cathepsins B and S when stimulated either by LPS or Il-6. In macrophages stimulated by LPS, a remarkable effect was observed with a dramatic and highly dose-dependent decrease of cysteine proteases B and S in both Raw 264 and THP-1 cells. In IL-6 stimulated macrophages, a moderate but significant decrease of cathepsins B and S was also observed. It may be interpreted as an important and pronounced effect in M1 macrophages and a less but nevertheless significant effect in M2 macrophages.

High levels of cathepsin protease activity are induced in the majority of macrophages in the microenvironment of primitive tumors and metastases during malignant progression [52]. Cathepsin proteases B and S are critical mediators, of the cancer-promoting functions of TAMs, with a significant association between increased levels of these cathepsins and tumor malignancy [23,53]. The increased activity of cathepsins was observed in tumor cells, macrophages, but also endothelial cells of the TME [52]. Inhibitors of cathepsins B and S could have antitumor but also anti-angiogenic and immune effects and cysteine proteases are now identified as key therapeutic targets in cancer and other diseases [58]. Interestingly, increased levels of cathepsin B were observed in chronic inflammation in the bowel disease macrophages and inhibition of cathepsins B (and L) was followed by a significant amelioration of colitis [55]. Other metallic compounds with other metals than Re, such as gold, ruthenium, palladium, iridium, and tellurium showed a decreased activity on cathepsin B [56]. Independently of its role on macrophage polarization, cathepsin B is a mediator of ferroptotic cell death, which is a necrotic form of cell death caused by inactivation of the glutathione system [57]. There is a great link between cathepsin B, oxidative stress and inflammation. Oxidative stress upregulates cathepsin B activity and by this way activates NLRP3 inflammasome [58]. On the other hand, there is a link between ferroptosis, NLRP3 inflammasome and ROS as shown by the use of luteolin preventing THP-1 macrophage pyroptosis by suppressing ROS production

[59]. It was shown that Re complexes could induce autophagy via the decrease of cathepsins B activities. In any case, inhibitors of cysteine proteases are promising drugs for anticancer treatment.

#### **4.4. Polarization of Macrophages**

It is known that the decrease of cathepsins may favor a shift from M2 to M1 macrophages. In fact, we observed a highly significant increase in the number of M1 macrophages, as noted by the increase in CD 80 biomarker in LPS stimulated macrophages treated by Re-diSe. We also observed a decrease in the number of M2 macrophages, at the low dose of 5  $\mu$ M Re-diSe, but only in Raw 264 macrophages stimulated by IL-6 and not in THP-1 cells. The two cell lines of macrophages are thus not similar.

Nevertheless, we can conclude that the Re-diSe drug increased the M1 and decreased the M2 expression, at least in Raw 264 macrophages. Since the classical M1 macrophage possesses antitumor activity, and M2 expression induces immune-suppressive effect, we can assume that besides its known inhibitory effects on the cancer cells [13], the Re-diSe drug may be endowed with immune effects that could counteract the resistance of cancer cells. It may be compared with Zoledronic acid that has been found to inhibit spontaneous mammary carcinogenesis by reverting macrophages from the M2 phenotype to the M1 phenotype [60]. Simvastatin was able to re-polarize TAMs, promoting M2-to-M1 phenotype, increasing TNF- $\alpha$ , attenuating TGF- $\beta$ , and suppressing Epithelial-mesenchymal transition (EMT) [61]. Ginsenoside Rh2 (G-Rh2), a monomeric compound extracted from ginseng had a potential to convert TAMs from M2 subset to M1 in the microenvironment and to prevent lung cancer cell migration in a study investigating the effects of a co-culture of RAW264.7 or THP-1 cells with Non-Small Cells Lung Carcinoma (NSCLC) [62]. Inducing specifically inflammation or decreasing M2 polarization in tumor tissues through the innate immunity system is new therapeutic strategy, utilizing the unique functions of macrophages [63–67].

#### **4.5. NO Production**

RediSe induced an increase in NO production in non-stimulated cells but a decrease in Raw-267 macrophages stimulated by LPS. There was nearly no effect on THP-1 macrophages. This difference on the NO production between Raw-267 and THP-1 cells has already been observed [68]. RAW 264.7 macrophages could be more relevant to study the LPS-induced COX-2 expression and NO production [48,69].

The contrast observed in the NO production in either non-stimulated or stimulated macrophages when treated by Re-diSe has to be emphasized, but needs to be elucidated.

Due to the increased number of macrophages of type 1, an excess of production of NO was expected, but not observed in our study in macrophages stimulated by LPS and treated by Re-diSe. In contrast, it is a decreased in the NO production that we noted in Raw-267 macrophages stimulated by LPS treated by Re-diSe at doses as low as 5  $\mu$ M. Similarly, a decreased production of ROS was previously observed in cancer cells overexpressing ROS levels upon Re-diSe exposure [13]. IL-25 is a Th2-like cytokine, promoting macrophage polarization toward the M2 phenotype, inducing intracellular ROS production in THP-1 derived macrophages and increasing the mitochondrial activity, and the mitophagy-related proteins [70]. Decreasing the M2-type of macrophages should therefore decrease their ROS production. On the other hand, ROS are also essential for TAMs to acquire a M2-like phenotype [71]. Scavenging ROS selectively could inhibit M2 macrophage polarization [72]. Re-diSe could still be considered as an anti-oxidant on ROS and RNS production but in cells producing on excess these radicals.

Other consequences of the increase on M1-type macrophages than the production of NO should be investigated in relation to other roles of M1 macrophages, like the antigen-processing, and the interaction with other immune cells. M1/M2-type macrophages direct T cells toward Th1-or Th2-like activities, respectively [73-74]. Th1-like cytokines stimulate specific cytolytic T cells and activate more M1 macrophages. In contrast, macrophages in the M2-like mode stimulate T cells to produce Th2-like cytokines (IL-4 and TGF- $\beta$ ) that cause B cell proliferation and antibody production and further amplify M2 responses [74]. It was shown that Re-diSe significantly decreased the production of TGF- $\beta$ , VEGF-A and IGF-1 by MDA-MB231 cancer cells [14] and the combined effects on the production of these cytokines with the decrease of cysteine proteases in both macrophages and cancer cells may amplified the antitumor effect and anti-angiogenic effects of Re-diSe.

Finally, the decreased effects of Re-diSe on the production of cathepsins B and S may be the main mechanism of action. The effect of Re-diSe on the cathepsins could be explained by a direct binding of Re on the thiol (SH) group of cysteine in the active site of the cysteine proteases in a similar manner that the complex reacted with cysteine or GSH. The reaction of dipyridine or phenanthroline rhenium complexes with thiol containing amino acids and peptides is well documented [11, 35, 36]. Such process occurred without decomplexation of the diimine ligand. In the present case, our results showed that the reaction with thiols first induced the release of the diselena-azelaic acid ligand. The liberated unhindered rhenium fragment is likely to easily accommodate in the active site of cysteine proteases as evidence by the formation of small clusters with glutathione. The enzymatic activity of the cysteine proteases will be inhibited. However, there is a need to verify the binding of the Re atom with cysteine proteases and not only with the thiol of N-acetylcysteine and GSH.

## **CONCLUSION**

The increased M1 phenotype of the macrophages is considered as favorable to kill the cancer cells, while the M2 phenotype is associated with immuno-resistance and tumor progression. As the Re-diSe drug increased the M1 and decreased the M2 phenotypes, it represents a new immuno-therapeutic approach. The NO production by the stimulated M1 macrophages by LPS was not increased by the Re-diSe, and even decreased in one kind of macrophage. Re-diSe remains to be considered rather as an anti-oxidant than a pro-oxidant drug as previously shown. The other known effects of M1 macrophages have thus to be explored in presence of Re-diSe, especially phagocytosis, presentation of the tumor antigens and interaction with other immune cells (T cells and NK cells).

The investigation of the role of Re-diSe on phagocytosis/autophagy has to be considered via its effects on the polarization of macrophages, but also as it is known that Re complexes could induce autophagy via the decrease of cathepsins cysteine proteases activities. The investigation of the role of Re-diSe on phagocytosis/autophagy will be a priority for further research.

The binding of Re with the thiols has been demonstrated and the consequences of the production of these adducts opens the door to new intensively researches.

## **LIST OF ABBREVIATIONS**

EB = Ethidium Bromide

IFN $\gamma$  = Interferon-gamma

LPS = Lipopolysaccharide

NO = Nitric Oxide

NOS = Nitric Oxide Synthase

TAMs = Tumor-associated Macrophages

TME = Tumor Microenvironment

TNF- $\alpha$  = Tumor Necrosis Factor-alpha

## **HUMAN AND ANIMAL RIGHTS**

No animals/humans were used for studies that are basis of this research.

## **CONSENT FOR PUBLICATION**

Not applicable.

## **AVAILABILITY OF DATA AND MATERIALS**

The data and supportive information are available within the article.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise. Philippe Collery has been designed as an inventor of a patent on “Rhenium complexes and their pharmaceutical use”. He is the manager of the Society for the Coordination of Therapeutic Researches, which is owner of the Intellectual Property Rights.

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## SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher’s website along with the published article.

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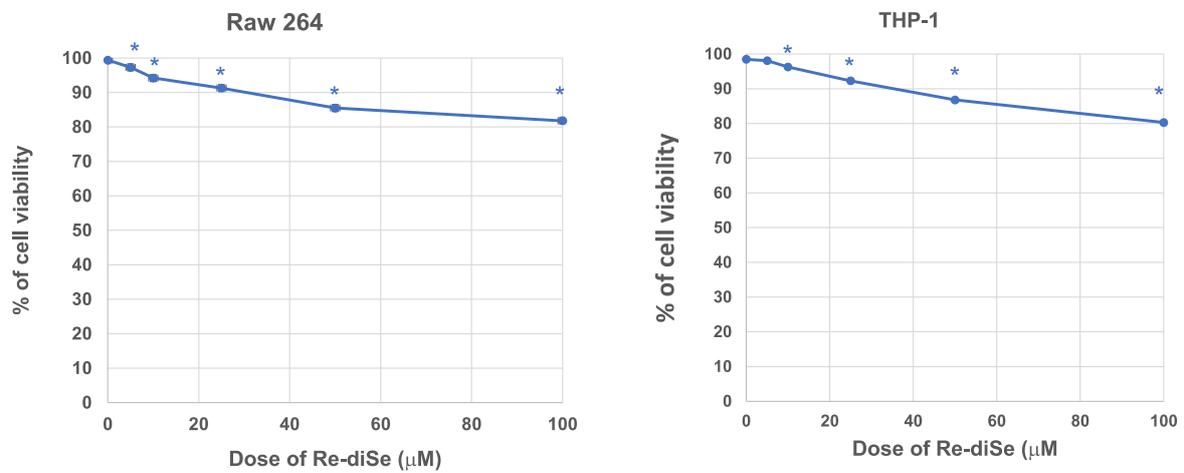
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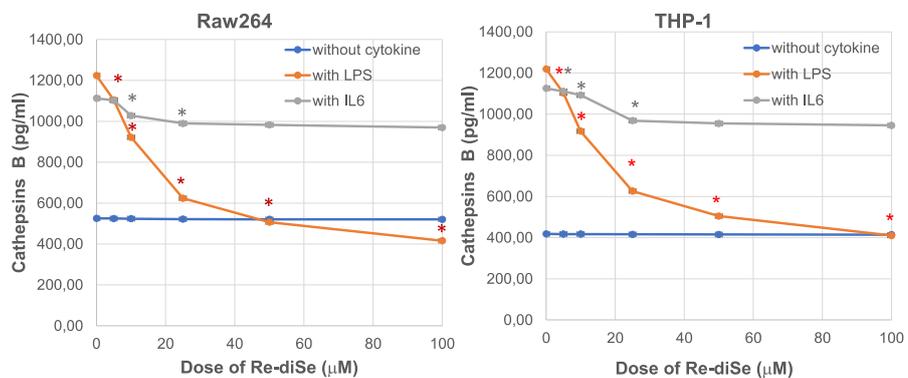
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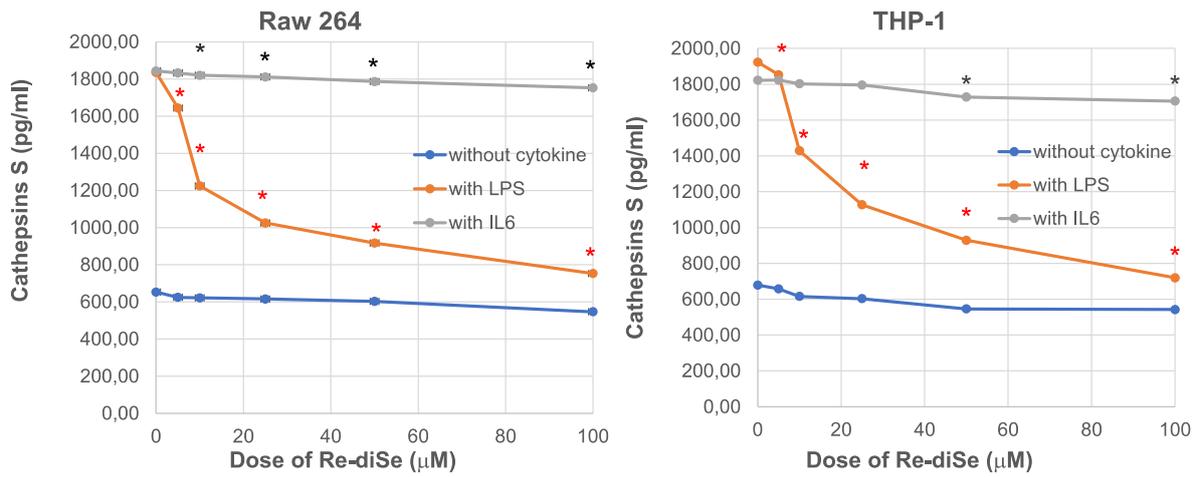
## FIGURES



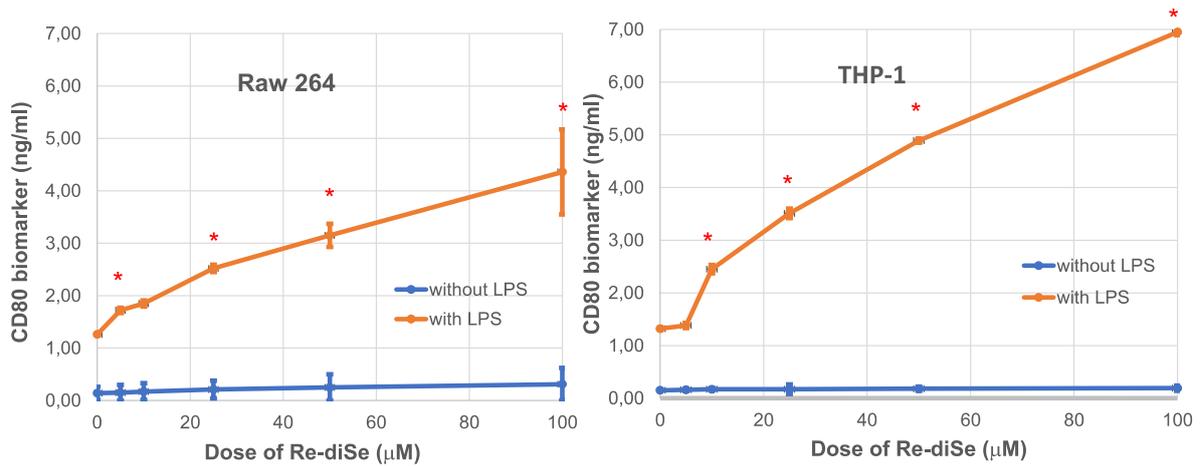
**FIGURE 1:** dose-dependent decrease of the cell viability of Raw 264 and THP-1 non-stimulated macrophages treated by Re-diSe for an exposure time of 120h. (\***P** <0.05: Results compared to the nearest lower value).



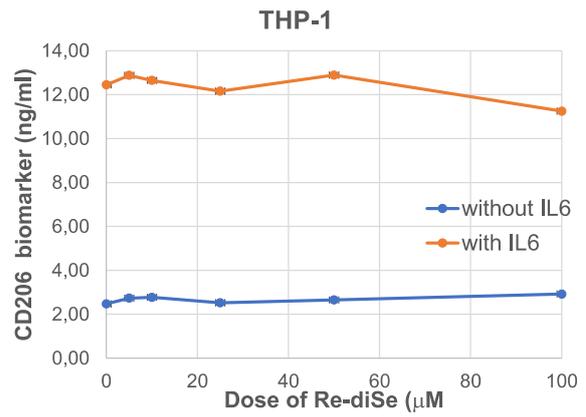
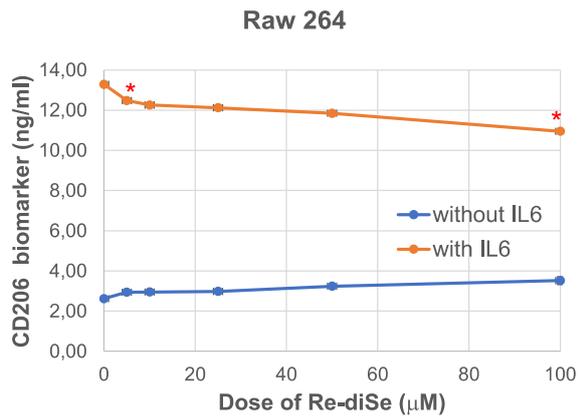
**FIGURE 2:** dose-effect of Re-diSe on the amount of cathepsins B in Raw 264 and THP-1 macrophages. \***p** <0.05: result compared to the nearest lower value.



**FIGURE 3:** dose-effect of Re-diSe on the levels of cathepsins S in Raw 264 and THP-1 macrophages. \*p < 0.05: result compared to the nearest lower value.

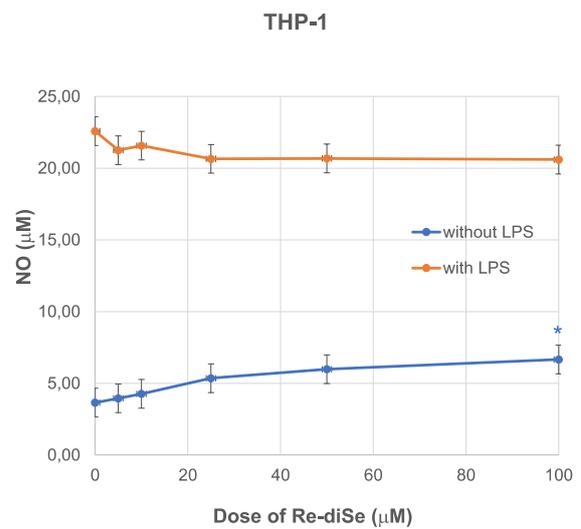
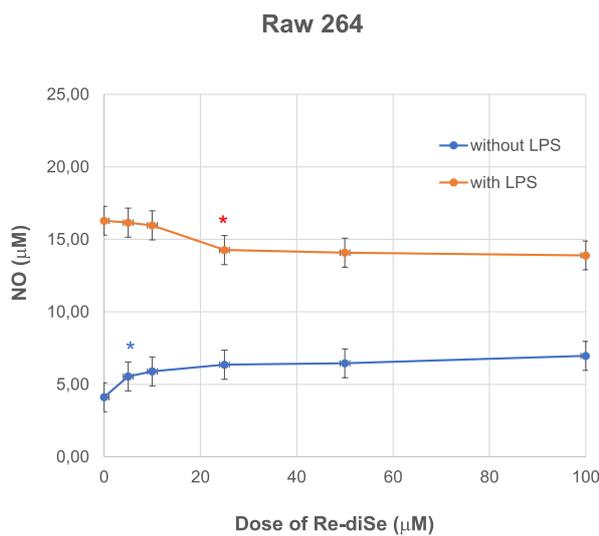


**FIGURE 4:** dose-effect of Re-diSe on the expression of CD80 markers in Raw 264 and THP-1 macrophages. \*p < 0.05: result compared to the nearest lower value.



**FIGURE 5:** dose-effect of Re-diSe on the expression of CD 206 markers in Raw 264 and THP-1 macrophages.

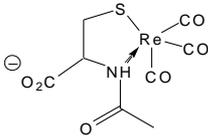
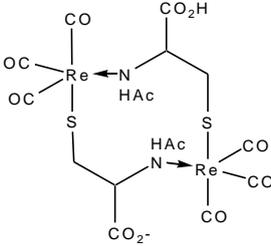
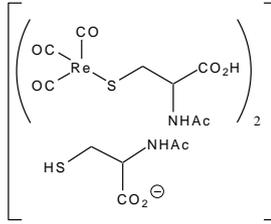
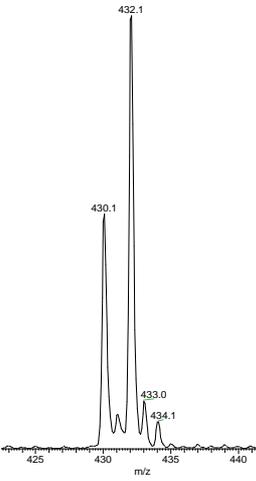
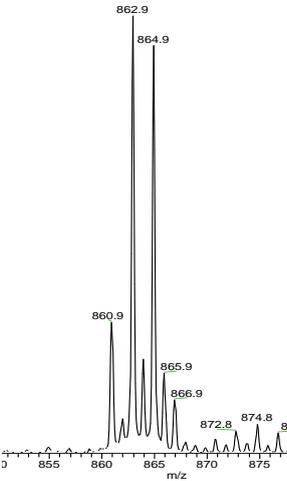
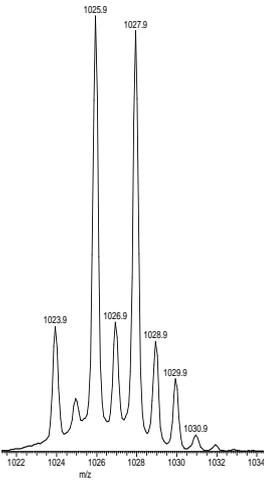
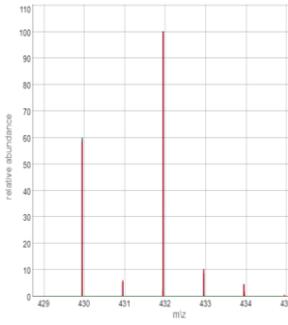
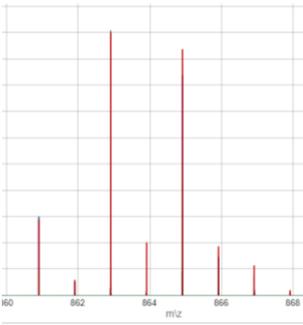
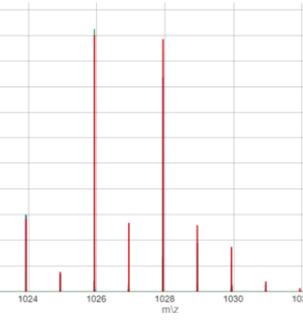
\*p <0.05: result compared to the nearest lower value.



**FIGURE 6:** dose-effect of Re-diSe on the NO quantity in Raw 264 and THP-1 macrophages stimulated or not

by LPS. \*p <0.05: result compared to the nearest lower value.

**TABLES**

Canonic formula	$C_8H_7NO_6ReS^-$	$C_{16}H_{15}N_2O_{12}Re_2S_2^-$	$C_{21}H_{24}N_3O_{15}Re_2S_3^-$
Molecular mass	431.90	862.92	1025.90
Putative structure			
Observed isotopic pattern			
Calculated Isotopic pattern			

**TABLE 1.** Structure, observed and calculated patterns of the most abundant ions in the reaction of Re-diSe with *N*-acetyl cysteine.

Canonic formula	$C_{13}H_{15}N_3O_9ReS^-$	$C_{21}H_{24}N_3O_{15}Re_2S_3^-$
Molecular mass	576.1	1459.2
Putative structure		
Observed isotopic pattern		
Calculated isotopic pattern		

**TABLE 2.** Structure, observed and calculated patterns of the most abundant ions in the reaction of Re-diSe with *GSH*.

#### SUPPLEMENTARY MATERIAL

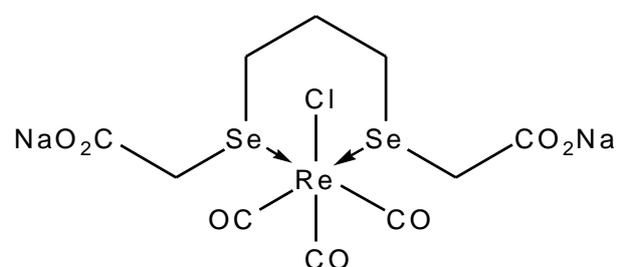


Figure S1. Structure of the rhenium complex (Re-diSe)

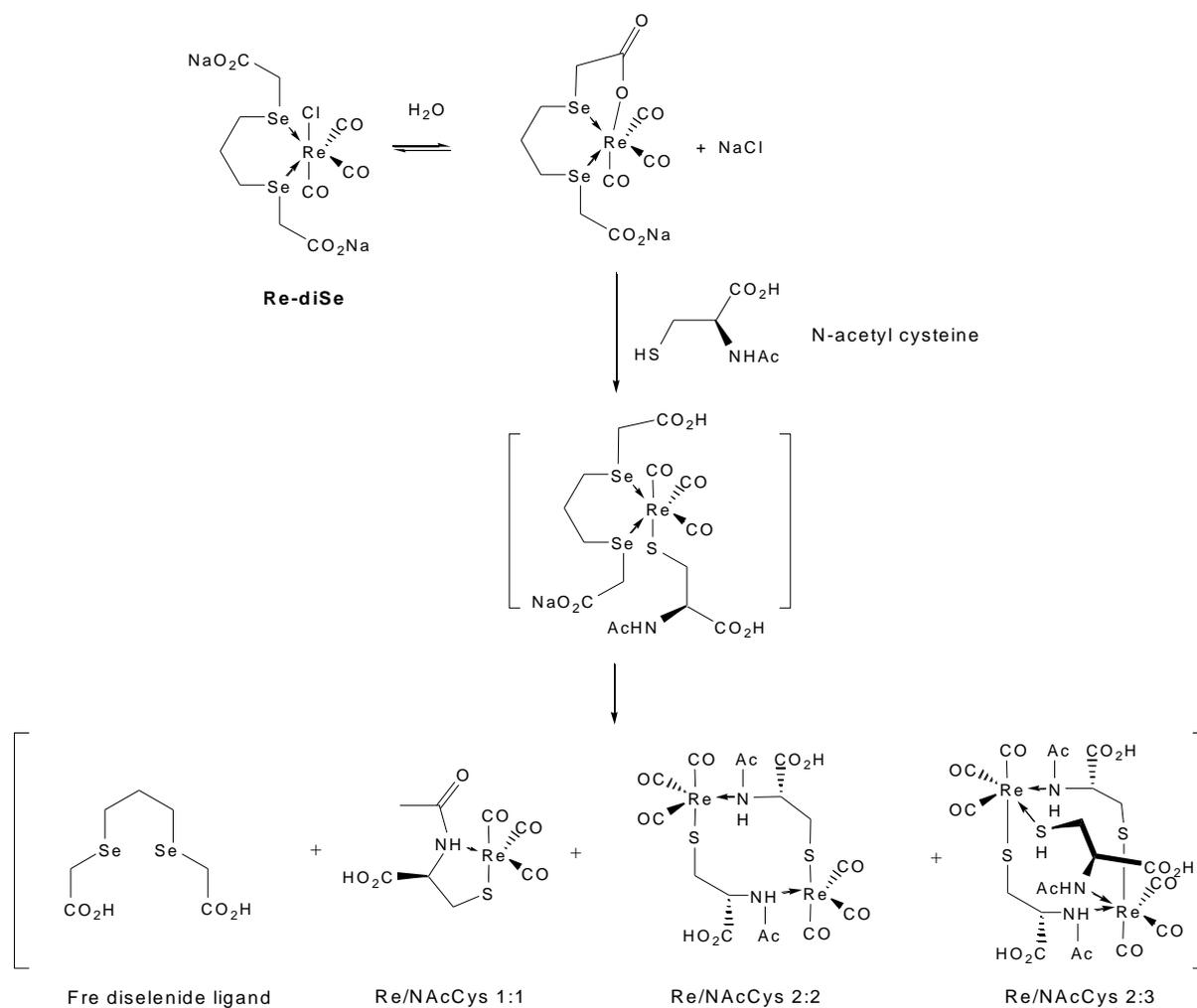


Figure S2. Presumed mechanism of the reaction of Re-diSe with N-acetyl cysteine and putative structure of the observed ions

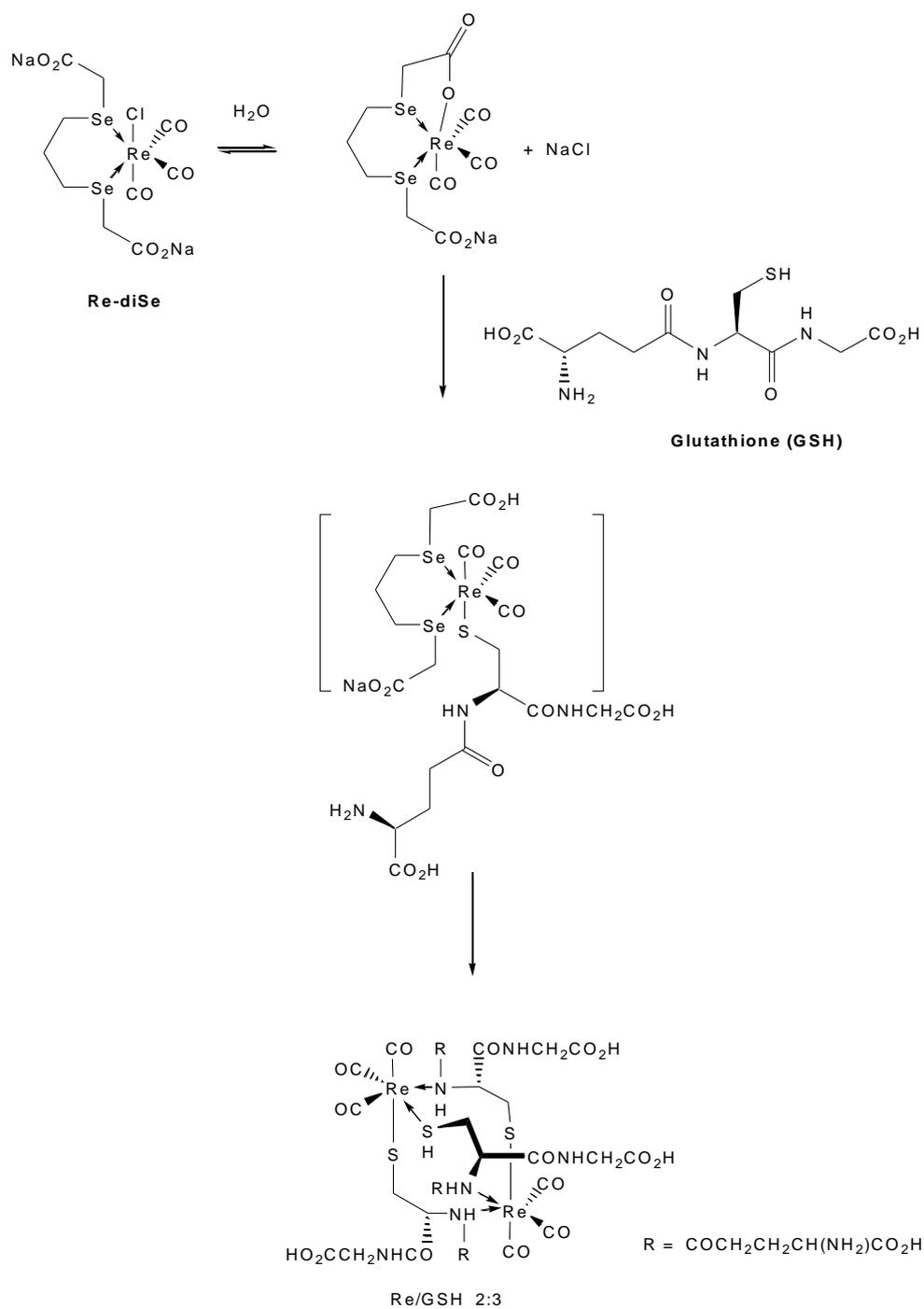


Figure S3. Presumed mechanism of the reaction of Re-diSe with glutathione and putative structure of the major observed ion