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Effects of Rhenium(I)-diselenoether and of its Diselenide Ligand on the Production of Cathepsins B and S by MDA-MB231 Breast Malignant Cells

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Abstract. *Background/Aim: Rhenium(I)-diselenoether (Re-diSe) is a drug under development for the treatment of metastatic cancers, with selective inhibitory effects on MDA-MB231 cancer cells compared to normal HEK-293 cells, and with greater effects than its diselenide (di-Se) ligand. Rhenium (Re) compounds inhibit cathepsins, which are important proteolytic enzymes in cancer. This study investigated the effects of Re-diSe and di-Se on the production of cathepsins B and S in MDA-MB231 malignant and HEK-293 normal cells and their inhibitory effects following treatment with different doses for 72 h. Materials and Methods: Elisa tests were used to assay the amount of cathepsins B and S in the medium of cultures. Results: Re-diSe, but not diSe affected the viability of malignant cells and the expression of cathepsins B and S. Conclusion: To the best of our knowledge, this is the first demonstration that Re-diSe may decrease the production of cathepsins B and S in cancer cells at doses as low as 10 μM.*

Cathepsins have a role in cancer development (1) and in tumor-associated immune cell functions (2). Since 2006, it is known that rhenium (Re) compounds inhibit the activity of cathepsin B (3). This was confirmed 2 years later by Fricker *et al.* (4). Among cathepsins, cysteine proteases are targets of metal-based drugs (5).

Rhenium(I)-diselenoether (Re-diSe) is a drug under preclinical development for the treatment of metastatic triple-negative breast cancer, with selective inhibitory effects on MDA-MB231 cancer cells compared to normal HEK-293 cells. Re-diSe displayed greater effects than its free diselenide (di-Se) ligand, emphasizing the role of the Re metal (6, 7). The selective decreased expression of ROS, TNF-α, VEGF, TGF-β in MDA-MB231 cancers cells in comparison with normal HEK-293 cells after treatment with Re-diSe has also been observed, but no study has yet been performed on cathepsins. Therefore, this study aimed to study the expression of cathepsins B and S in Re-diSe- and diSe-treated MDA-MB231 cancer cells and normal HEK-293 cells and to correlate the results obtained with the inhibitory effects of the drugs.

Materials and Methods

Synthesis of the drugs. The procedure of synthesis of the diSe ligand and the Re-diSe complex have been previously reported (6, 8).

Cell lines and reagents. Cell lines were purchased from the National Centre for Cell Science (NCCS), Pune, India and maintained in a CO_2 incubator as per the standard protocol. Dimethyl sulfoxide (DMSO), Dulbecco's modified eagle medium (DMEM), 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), and ethidium bromide (EB) were purchased from Sigma Aldrich (St. Louis, MO, USA). ELISA kits for the biomarkers were purchased from ELabscience (Houston, TX, USA).

Assays of the inhibitory effects. The inhibitory effects were studied by the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

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Key Words: Cathepsins, cysteine proteases, rhenium, selenium.

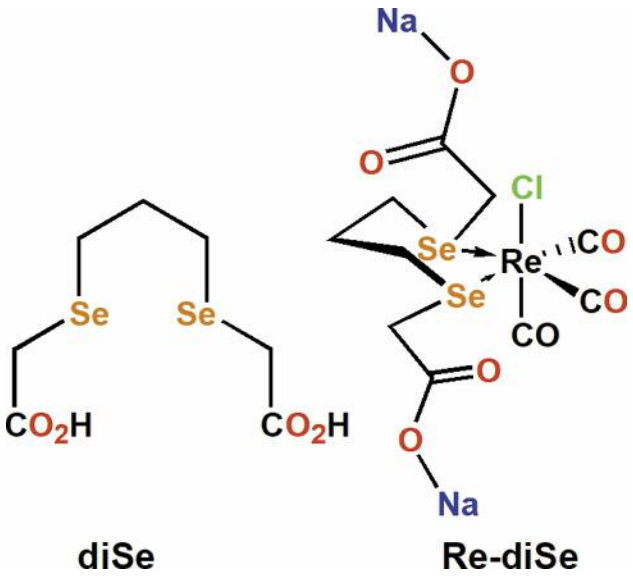

Figure 1. *Structure of the rhenium complex Re-diSe and its 3,7-diselena-nonanedioic acid ligand (diSe).*

tetrazolium reduction assay (MTT test). They were assayed in hormone-independent MDA-MB231 breast cancer cells and in the normal human embryonic kidney cell line (HEK-293).

The inhibitory effects were assayed after exposure of cells to the drug for 72 h at doses of 5, 10, 25, 50, and 100 μM. We compared the effects of the Re-diSe compound *versus* its di-Se ligand. The drug concentration leading to 50% inhibition of the proliferation of cells (IC_{50}) was defined.

Assays of cathepsins. Cathepsins B and S were assayed by ELISA tests in the culture medium, reflecting their release by the cells, but not directly in the cells, where the changes are far too rapid to be detected by these methods.

Statistical analysis. Results are expressed as mean±SD of triplicate experiments. The statistical analysis was performed using the SPSS software (IBM, Chicago, IL, USA). The comparison of the efficacy of the Re-diSe drug to inhibit cancer and normal cell lines was analyzed using the Mann-Whitney *U*-tests. Results were considered statistically significant at $p<0.05$.

Results

Structure of Re-diSe. The rhenium(I)-diselenoether complex depicted in Figure 1 features a central *fac*-[$Re(CO)_3$)] core coordinated by two Se atoms bearing to acetic acid appendages. Conversion of the two carboxylic acid functions into water-soluble disodium salt markedly facilitates its application in biological systems. This complex was obtained by simple ligand exchange reaction of pentacarbonylchlo-rorhenium (I) with 3,7-diselena nonanedioic acid (diSe, Figure 1).

Inhibitory effects. The IC_{50} of Re-diSe in malignant cells was 50 μM and was not reached with diSe. The selectivity of Re-diSe for malignant cells was confirmed by the less than 10% inhibitory effect of this dose (50 μM) in normal HEK-293 cells. A significant decrease in the cell proliferation of normal cells was only observed with the 100 μM dose of Re-diSe. The effect of Re-diSe was dose-dependent, with a significant decrease in the proliferation of malignant cells from 10 μM, with significantly increased effects at higher doses. Treatment with 50 μM di-Se ligand inhibited proliferation of malignant cells by less than 10%, as observed in normal cells. The diSe compound had no significant effect on cell viability following treatment for 72 h, even at the dose of 100 μM. The results are shown in Figure 2.

Expression of cathepsins

Cathepsin S. The levels of cathepsin S were significantly higher in the medium of malignant MDA-MB231 cells than in the medium of normal HEK-293 cells after 72 h of culture in non-treated cells.

A significant decrease in cathepsin S was observed following treatment of malignant cells with 10 μM of Re-diSe and the effects were dose-dependent (Figure 3). The decrease in cathepsin S was only significant in normal cells treated with 50 μM Re-diSe, which was the IC_{50} dose for malignant cells. At the dose of 10 μM, the levels of cathepsin S decreased in the medium of malignant cells from 501±23 pg/ml (non-treated cells) to 457±2 pg/ml ($p<0.05$) and from 325±34 pg/ml (non-treated cells) to 316±30 pg/ml in the medium of normal cells (N.S). The diSe drug had no significant effect on the levels of cathepsin S.

Cathepsin B. The levels of cathepsin B in non-treated cells were significantly higher in the medium of malignant MDA-MB231 cells than in the medium of normal HEK-293 cells after 72 h of culture.

A significant decrease in cathepsin B was observed at the dose of 10 μM/l of Re-diSe both in normal and malignant cells compared to non-treated cells (Figure 4). The effects were dose-dependent. At the dose of 10 μM/l Re-diSe, the levels of cathepsin B decreased from 5619±68 pg/ml to 5012±80 pg/ml ($p<0.05$) in the medium of malignant cells and from 4538±28 pg/ml to 4231±38 pg/ml in the medium of normal cells ($p<0.05$). The diSe had no significant effect on the levels of cathepsin B.

Discussion

Cathepsins are classified according to the amino acids of their active sites in three classes: aspartic, cysteine, or serine proteases (9). The nucleophile is provided by a sulfhydryl group of cysteine in cysteine cathepsins.

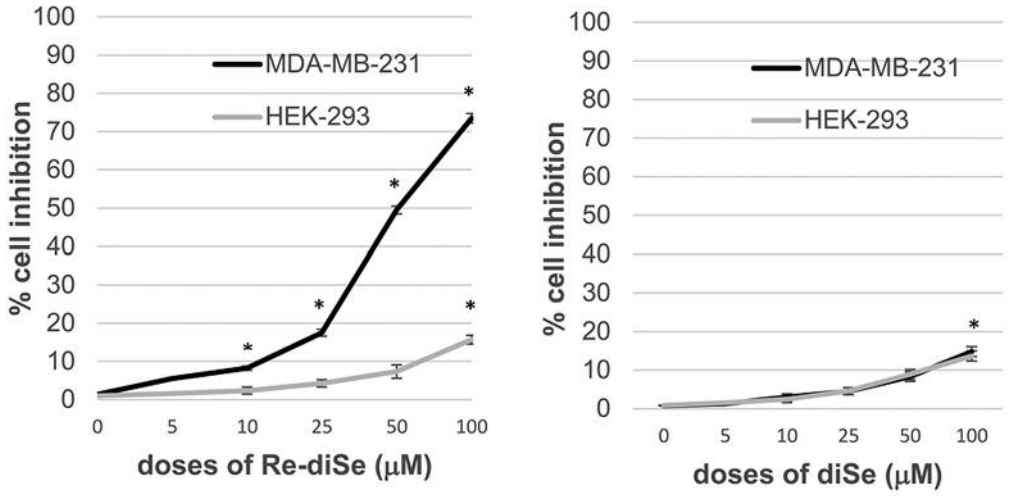

Figure 2. *Dose effects of Re-diSe and diSe on the proliferation of MDA MB-231 and HEK-293 cells (*p<0.05 versus non-treated cells).*

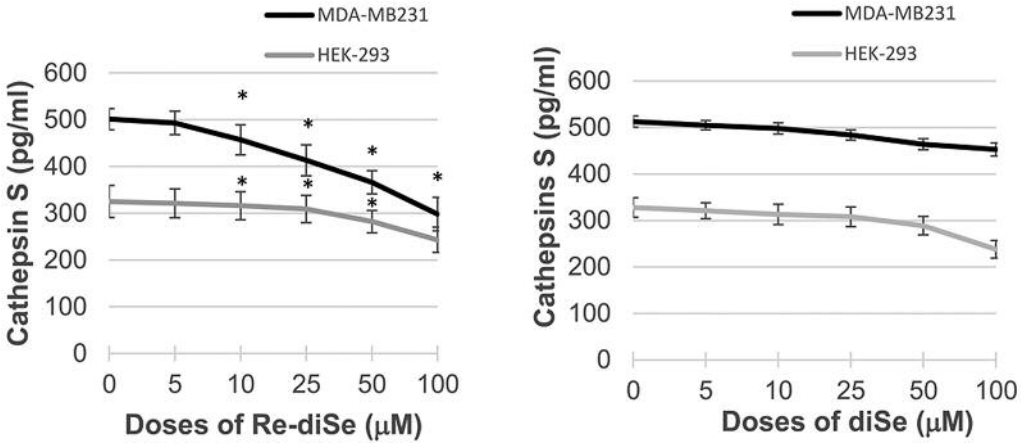

Figure 3. *Dose-effects of Re-diSe and diSe on the production of cathepsin S by MDA-MB231 cancer cells and HEK293 normal cells (*p<0.05 versus non-treated cells).*

There are two distinct subfamilies of cysteine proteases, cathepsin-L-like (cathepsins L, V, K, S, W, F, and H) and cathepsin-B-like proteases, which can be distinguished by the structure of the prodomain and the mature domain, with two conserved motifs (ERFNIN and GNFD) in the prodomain of the cathepsin L subfamily, while the ERFNIN motif is lacking in cathepsins B, C, O, and X (10). Cathepsins B and L are papain-like enzymes. Their zymogen inactive forms have to be activated to obtain the enzymatic function. Comparisons between procathepsins B and L have been well described by Verma *et al*. (10). Cathepsin S was chosen in our study as a member of the cathepsin L subfamily. Cathepsin B was chosen as a member of the other subfamily, with a characteristic feature due to an "occluding loop", which provides carboxydipeptidase activity.

We demonstrated in this study that Re-diSe, but not diSe, selectively inhibited the proliferation of breast cancer cells that over-express cathepsins B and S, compared to normal cells.

We also observed a dose-dependent decrease in both cathepsin B and S in malignant cells, from the low dose of 10 μM Re-diSe, whereas the IC_{50} was 50 μM. There is an increased expression of cathepsins in cancers (11) and a higher expression of cathepsins in MDA-MB-231 cancer cells *versus* normal HEK-293 cells was found in this study.

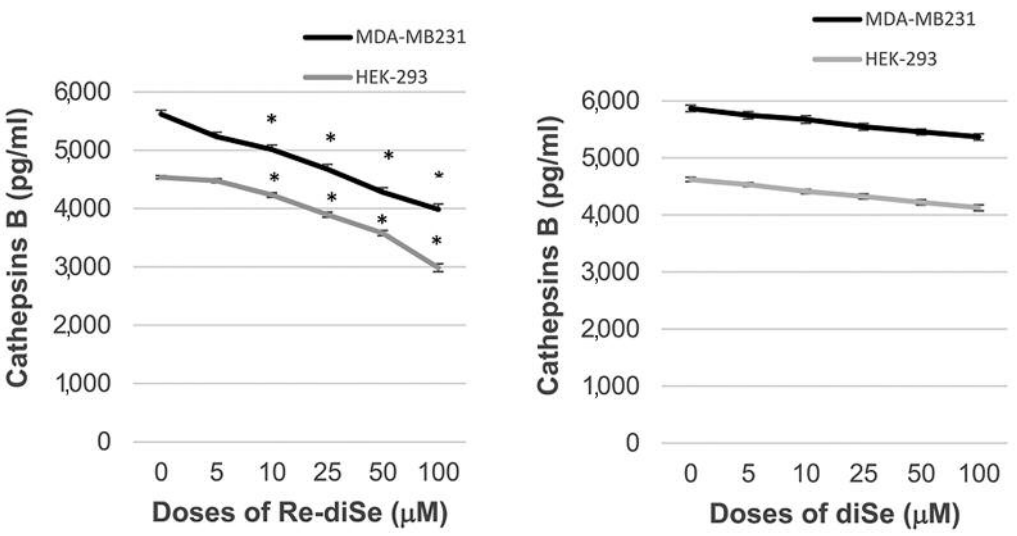

Figure 4. *Dose-effects of Re-diSe and diSe on the production of cathepsin B by MDA-MB231 cancer cells and HEK293 normal cells (*p<0.05 versus non-treated cells).*

The role of cathepsin B, which is over-expressed in a wide variety of human cancers, has been reviewed by Mijanovic *et al*. (1). The decreased production of cathepsins B and S may have an antitumor effect; several publications have reported that inhibition of the activity of cathepsins B and S could induce antitumor effects (11-15).

We also observed a significant decrease in cathepsin B and S upon Re-diSe treatment of normal HEK cells; further experiments are needed to show whether this occurs in the immune cells of the microenvironment (dendritic cells, B- and T-cells, and macrophages). Cathepsin S is one of the major cysteine proteases. It is expressed in the lysosome of antigen presenting cells (9), with specific roles such as MHC class II antigen presentation, and cathepsin S inhibitors have been proposed as novel immunomodulators (16). However, cathepsin S can promote degradation of damaged or unwanted proteins in the endo-lysosomal pathway (17).

Macrophages may have opposing functions, defined as M1 (TH1-driven) and M2 (TH2-driven). These states are reversible, due to their functional plasticity, by changes in the characteristics of the microenvironment (18). Inhibition of cathepsins B and S, using a specific inhibitor, suggest a shift from M2 to M1-like phenotype, with an increased expression of autophagy- and lysosome-associated marker genes, changes in lysosomal activity, fatty acid metabolism, synthesis of pro-inflammatory mediators and reduced adenosine triphosphate (ATP) levels (19). Therefore, re-polarization of tumor-associated macrophages from M2 towards an M1-like phenotype offers great potential for cancer therapy, especially for overcoming drug resistance and counteracting the immuno-suppressive effects of cancer (18).

Conclusion

The role of the Re atom has been emphasized by the significant effects of Re-diSe, but not of diSe, on cell proliferation and on the production of cathepsins. The decreased production of cathepsins B and S by Re-diSe may either have a direct action on cancer cells or indirect effects through its effects on immune cells and on the extracellular matrix. Therefore, further studies are required to investigate the effects of the Re-diSe on immune cells of the tumor microenvironment.

Conflicts of Interest

Philippe Collery is the manager of the Society for the Coordination of Therapeutic Research, and patents on rhenium compounds for pharmaceutical use belong to this Society. The other Authors declare that they have no conflict of interest related to this study.

Authors' Contributions

The idea of studying the effects of the Re compound on cathepsins was of Philippe Collery, who is involved in the development of this drug. The full protocol was elaborated by Vijay Veena. Didier Desmaele, synthesized the Re-diSe and diSe compounds. The experiments were performed by Vijay Veena in her laboratory, in collaboration with Adhikesavan Harikrishnan and Basavegowda Lakshmi. Vijay Veena and Adhikesavan Harikrishnan performed the statistical analysis. Vijay Veena and Philippe Collery were the main contributors to the writing of the manuscript.

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