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FRONTIERS IN LIFE SCIENCE

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CNBSF <Irreversible GST inhibitor>

Catalog NO. FDV-0031

Research use only, not for human or animal therapeutic or diagnostic use.

Product Background

Glutathione *S*-Transferases (GSTs) are widely conserved in nature from bacteria to plants and animals. In human, over 20 members are identified and classified into three categories: cytosolic, mitochondrial, and membrane-bound microsomal members. Cytosolic GSTs consist of 6 subfamilies including α (GSTA), μ (GSTM), π (GSTP), θ (GSTT), ο (GSTO) and ζ (GSTZ). Mitochondrial member is κ (GSTK) and microsomal members are MGSTs and membrane associated proteins in eicosanoid and glutathione metabolism (MAPEGs). GSTs are phase-II detoxification enzymes and commonly play an important role in detoxification of hydrophobic and electrophilic compounds including endogenous toxic metabolites or xenobiotics by conjugating with glutathione (GSH) to produce glutathione-conjugate (GS-conjugates) (Figure 1). Generally, GSTs have two types of substrate-binding site, called G-site and H-site, for GSH and hydrophobic substrate (xenobiotics), respectively. When GSTs bind to GSH as the first substrate, GSTs catalyze and stabilize thiol group of GSH as thiolate anion. Once hydrophobic and electrophilic xenobiotics bind to GSTs as the second substrate, GSTs transfer them to GSH to form GS-conjugates. GS-conjugates are released from GSTs and quickly exported to extracellular space by multidrug resistance-associated protein (MRP) transporters. Through the above processes, GSTs detoxify toxic compounds.

As many studies suggested expression level of GSTs are significantly increased in cancer cells, GSTs are considered as anticancer drug-resistant enzymes in malignant cancer cells through the neutralization of drugs. Inhibition of GST activities is one of the promising strategies to improve drug efficiency in cancer cells. Some GST inhibitors have been developed so far, however, conventional inhibitors including ethacrynic acid (EA), a representative GST inhibitor, were based on the competition with GSH. In general, competitive inhibitors are usually reversible and not sufficient in cells because of high concentration around mM order of GSH in cells. Although several irreversible inhibitors were also discovered and show potent inhibition *in vitro*, these compounds have low membrane-permeability and are not good at live cell experiments. To overcome conventional problems, highly membrane-permeable and irreversible inhibitors are desired.

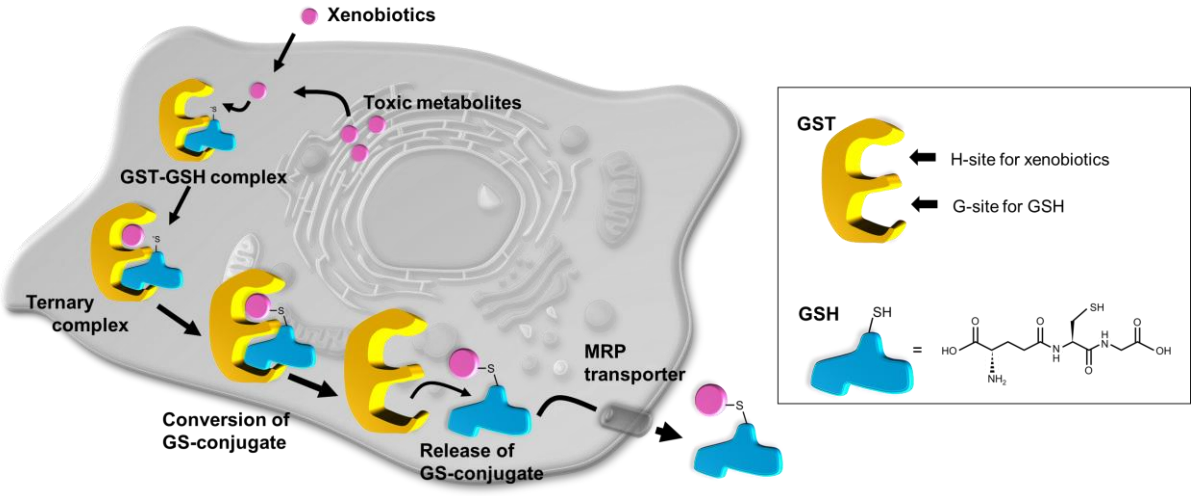


Figure 1. Overview of the detoxification process of GSTs

2-**c**hloro-5-**n**itro**b**enzen**s**ulfonyl **f**luoride (CNBSF) is a novel type of irreversible GST inhibitor reported in 2019 (Ref.1). CNBSF is membrane-permeability and capable of entering into cytosol. Once CNBSF incorporated into cell, GSTs catch CNBSF in H-site as a hydrophobic xenobiotics. Subsequently GSTs convert CNBSF to GS-conjugated CNBSF, called GS-5NBSF. In the case of human GSTP1, a member of π-type GST, Tyr108 residue in human GSTP1 quickly reacts with sulfonyl fluoride group of GS-5NBSF, fluoride anion was leaved, and form covalent bound between GST and substrate. In the result of above scheme, GSTs covalently bound inhibitor-complex are inactivated.

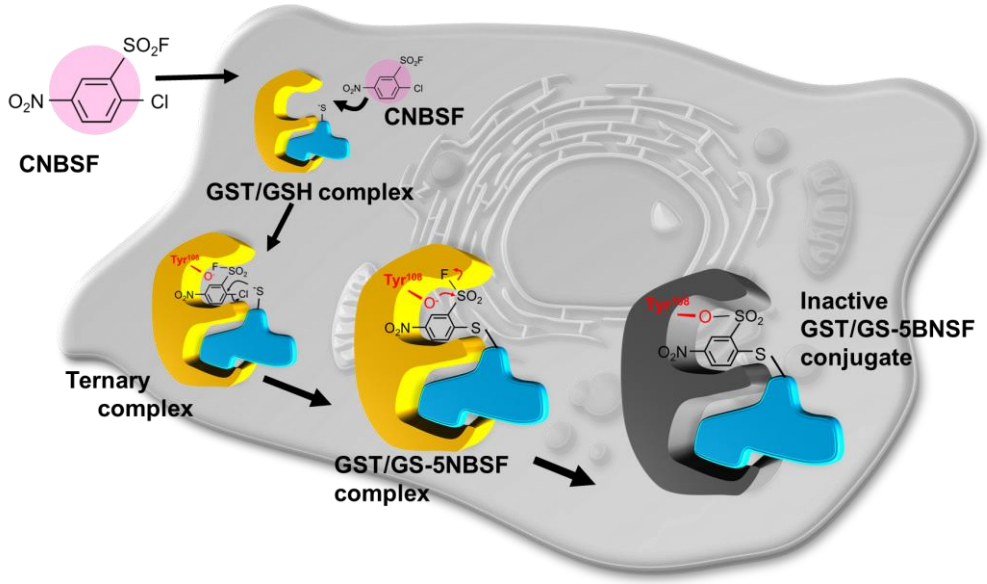
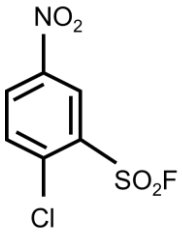


Figure 2. Inhibition mechanism of CNBSF

Description

Catalog Number: FDV-0031
Size : 10 mg
Formulation : $C_6H_3ClFNO_4S$
CAS No. : 3829-23-0
Chemical structure : See right figure
Molecular weight : 239.6 g/mol
Solubility : Soluble in DMSO



Reconstitution and Storage

Reconstitution : Stock solution in 100% DMSO.
Storage (solution) :
Store powder at -20°C.
After reconstitution in DMSO, aliquot and store at -20 °C. Avoid repeated freeze-thaw cycles.

Recommended concentration

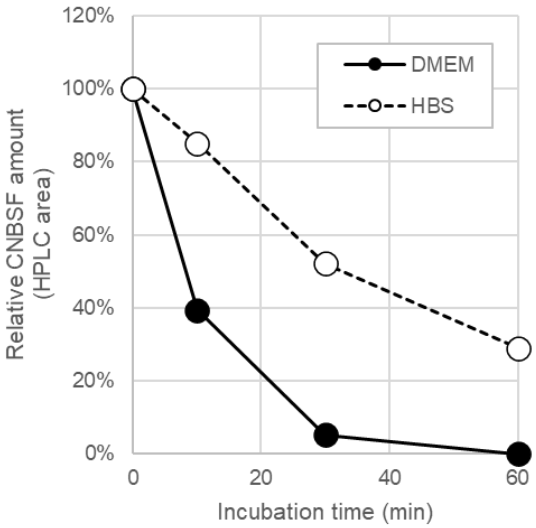
For cell-based experiments : 50-1000 μM

For *in vitro* experiments : 100-1000 μM

* For both cases, the concentration should be optimized by yourself.

Note for experimental setting

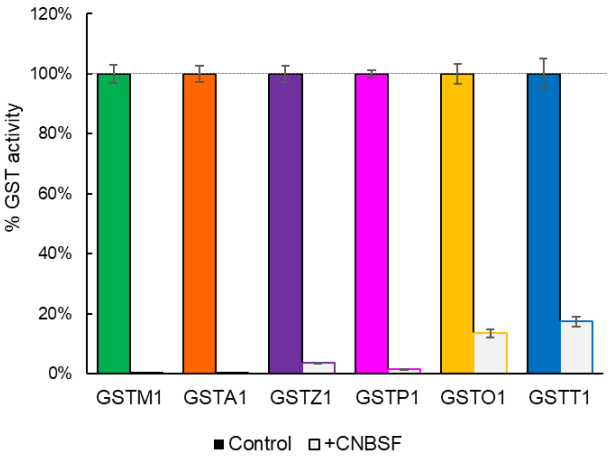
CNBSF can be stored in DMSO stock solution for long-term in less than -20°C . However, CNBSF shows highly chemical reactivity in aqueous solutions. Especially in culture media such as DMEM, CNBSF quickly reacts with various components of DMEM, resulting in its decomposition. For example, a stability of CNBSF in serum-free DMEM at 37°C is experimentally estimated, 60% degradation in 10 min and ~95% degradation in 30 min. Therefore, the use of inorganic salt buffers such as HEPES-buffered saline (HBS; *e.g.* 20 mM HEPES (pH 7.4), 107 mM NaCl, 6 mM KCl, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$) is recommended for live cell experiments. Hydrolysis of sulfonyl fluoride group of CNBSF is still observed gradually even in HBS (~50% hydrolysis in 30 min, ~70% hydrolysis in 60 min at 37°C in HBS). So, working solution in aqueous solution such as HBS should be prepared just before use to avoid hydrolysis of CNBSF. Based on degradation or hydrolysis of CNBSF in buffer or medium, long-term incubation (>1 hour) of CNBSF for cells may not effectivelly. If the use of medium is required for your experiments, please optimize CNBSF concentration such as 1 mM.



Reference data

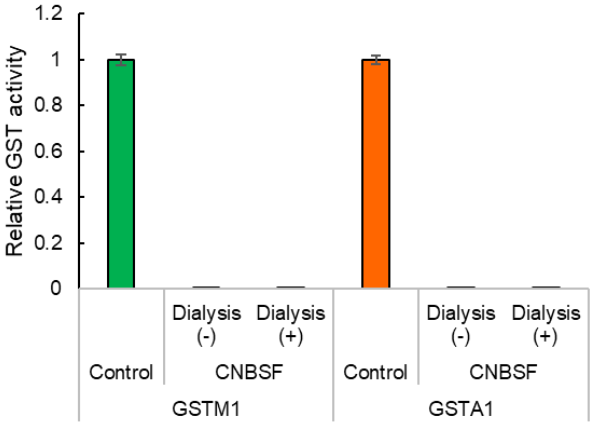
Broad inhibition activity for cytosolic GST members

Recombinant human or mouse cytosolic GST enzymes and reduced GSH (10 μM) were added into assay buffer (50 mM sodium phosphate (pH 7.4), 150 mM NaCl). DMSO for the control experiment or CNBSF (100 μM) was further added to them and incubated for 30 min. After then, a fluorescent GST activity assay reagent **CellFluor™ GST** (final 5 μM; Funakoshi Catalog No. #FDV-0030) was added and incubated for 30 min. Fluorescent intensity of each sample was measured by fluorescent plate leader (Ex 470±5 nm/Em 525±10 nm). CNBSF effectively inhibited all cytosolic GST enzymes tested here.



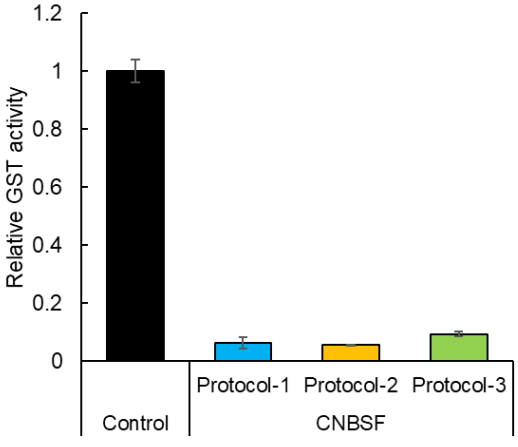
Irreversible inhibition activity *in vitro*

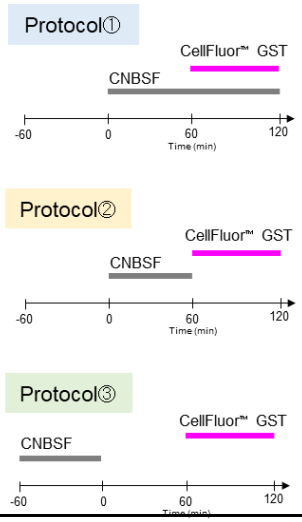
Recombinant human GSTM1 or GSTA1, GSH (final 100 μM) and CNBSF (final 100 μM) were added to PBS and incubated for 30 min at 37°C. Each sample was split into two vials and one vial was applied into micro-dialysis kit (1 kDa-cut off) for 30 min in PBS. After dialysis, reduced GSH (additionally 100 μM) and **CellFluor™ GST** (1 μM) were further added to each sample and incubated for 30 min. Neither GSTM1 nor GSTA1 activity was restored by dialysis after addition of CNBSF.



Irreversible inhibition activity *in cellulo*

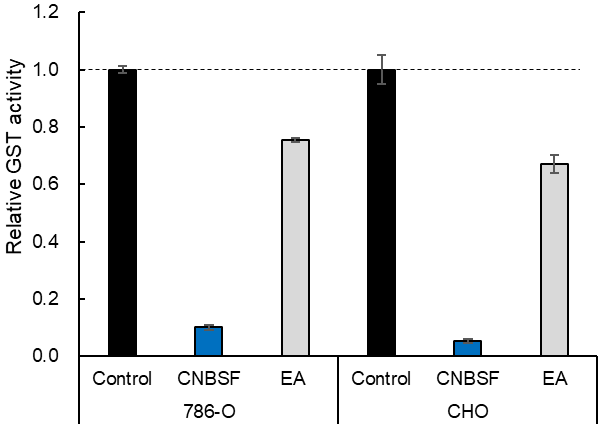
CHO cells were seeded in 96 well plate at 1 x 10^4 cell/well and cultured in DMEM containing 10% FBS for 20 hours. Then culture media were replaced with HBS, treated with 100 μM CNBSF and **CellFluor™ GST** in 3 different protocols shown in the right scheme. The fluorescent intensities were measured with a fluorescent plate reader (Ex 475±5 nm/ Em 525±10 nm). In all protocols, GST activities were highly suppressed, indicating that CNBSF irreversibly inhibits intracellular GSTs.





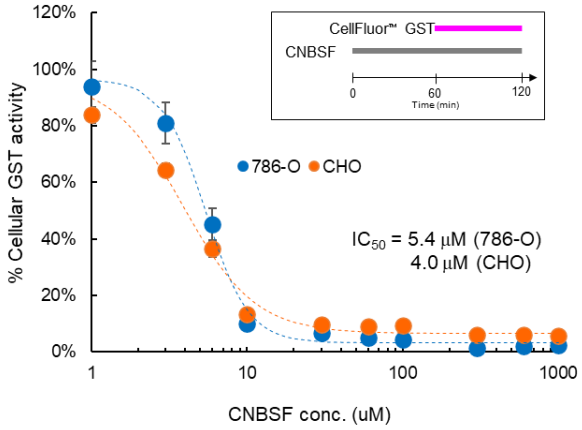
Comparison of intracellular GST inhibition between CNBSF and ethacrynic acid (EA) *in cellulo*

786-O and CHO were seeded in 96 well plate at 1 x 10^4 cell/well, cultured in DMEM containing 10% FBS for 20 hours. Then culture media were replaced with HBS, cells were incubated in the presence of 20 µM CNBSF or ethacrynic acid (EA) for 1 hour, and further treated with 2 µM **CellFluor™ GST** for 1 hour. Fluorescent intensities were measured with a fluorescent plate reader (Ex 475±5 nm/ Em 525±10 nm) without medium change, In both 786-O and CHO cells, CNBSF showed higher GST inhibitory activity than ethacrynic acid at 20 µM concentration.



Quantification of inhibitory activity of CNBSF *in cellulo*

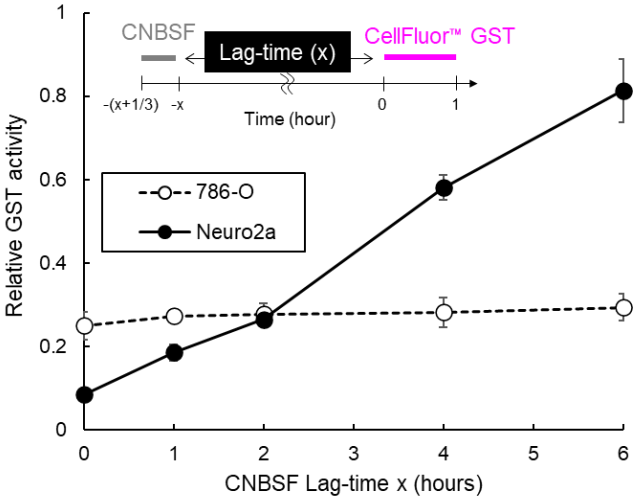
786-O and CHO cells were seeded in 96 well plate at 1 x 10^4 cell/well and cultured in DMEM containing 10% FBS for 20 hours. Then culture media were replaced with HBS, treated with 1-1000 µM CNBSF for 1 hour, and further treated with 2 µM **CellFluor™ GST** for 1 hour. The fluorescent intensities were measured with a fluorescence plate reader (Ex 475±5 nm/ Em 525±10 nm). Both 786-O and CHO cell lines showed similar IC_{50} values around 4-5 µM in cell-based assay.



Application data

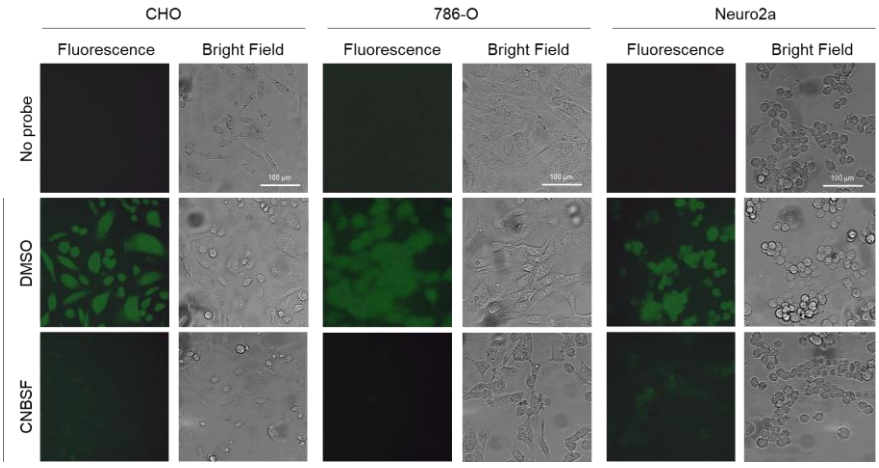
Estimation of recovery time of GST expression

786-O and Neuro2a were seeded in 96 well plate at 1 x 10^4 cell/well, and cultured in DMEM containing 10% FBS for 20 hours. Then the culture media were replaced with serum-and phenol red-free DMEM (DMEM(-)). After incubation in HBS containing 100 µM CNBSF for 20 min, the HBS media were returned to DMEM(-) and cultured for 0, 2, 4, and 6 hours. The cells were then simultaneously treated with DMEM(-) containing **CellFluor™ GST** (2 µM) for 1 hour. The fluorescent intensities were measured with a fluorescent plate reader (Ex 475±5 nm/ Em 525±10 nm). In 786-O cells, the inhibitory effect of GST was observed to continue for at least 6 hours, while a gradual recovery was observed over time for Neuro2a cells, indicating prompt rescue expression of GSTs .



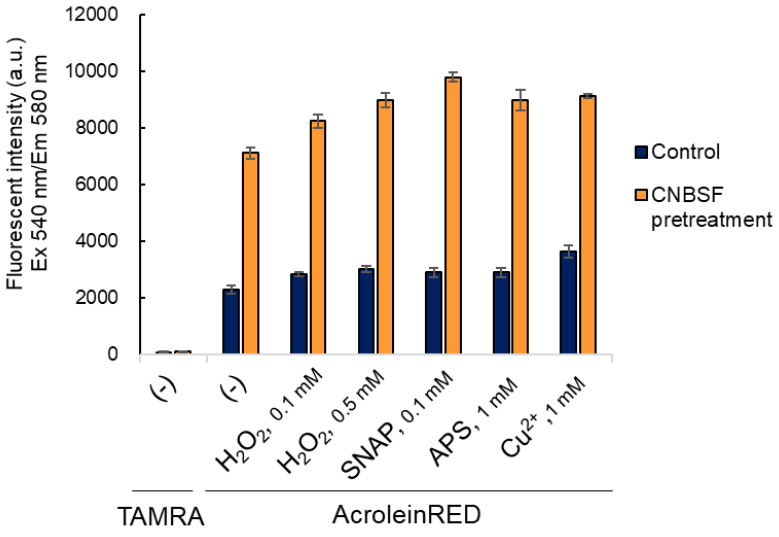
Fluorescent imaging

Three cell lines (CHO, 786-O, and Neuro2a) were seeded into glass bottom dishes and culture for 20 hours in 10% FBS-containing DMEM. Culture medium was replaced to serum- and phenol red-free DMEM and **CellFluor™ GST** (final 30 µM) was incubated for 10 min. Just before observation, the culture media were replaced to fresh serum- and phenol red-free DMEM and quickly observed by epi-fluorescent microscopy (Ex 435-475 nm/Em 530-543 nm). In all cell lines, green fluorescent signals were observed from inside of the cells. On the other hand, when 100 µM CNBSF was pretreated to cells for 30 min, the fluorescent signals were dramatically suppressed.



CNBSF promoted production of lipid-peroxidation (LPO) downstream metabolite

CHO cells were seeded in 96 well plate at 1 x 10^4 cell/well and cultured in DMEM containing 10% FBS for 20 hours. Then culture media were replaced with HBS and pretreated with 100 µM CNBSF for 1 hour. Cells were then washed with PBS, subsequently treated with lipid-peroxidation (LPO) initiators, H_2O_2 (0.1 mM or 0.5 mM), nitric oxide (NO) donor (SNAP; 0.1 mM), or radical initiator (APS; 1 mM) in HBS for 20 min or Cu^{2+} ($CuSO_4$; 1 mM) in HBS for 5 min. Cells were washed with HBS and AcroleinRED (2 µM), a cell-based detection reagent for acrolein (Funakoshi catalog No. FDV-0022), or TAMRA fluorescent dye as a negative control in HBS was treated for 30 min. Then, cells were washed 3 times with HBS to remove unreacted AcroleinRED or TAMRA, and fluorescent intensities were measured with a fluorescent plate reader (Ex 540±5 nm/ Em 580±10 nm). Under the control condition, each drug slightly increased acrolein production. Pretreatment of CNBSF dramatically increased acrolein production and CNBSF with each LPO initiators synergistically promotes acrolein production. These results suggest the physiologically generated or drug-induced acrolein was suppressed by the cellular GST activities.



Reference

1. Shishido *et al.*, *Chembiochem.*, **20**, 900-905 (2019) A covalent inhibitor for Glutathione S-Transferase Pi (GSTP1-1) in human cells.

Appendix: Proposal reaction mechanism of CNBSF to GSTs

Ref.1 proposed the reaction mechanism of CNBSF in the case of human GSTP1.

1) GSH and CNBSF are bound to G-site and H-site in GST, respectively. Tyr 7 of GSTP1 stabilizes thiolate anion of GSH and the thiolate anion of GSH attacks to CNBSF.
2) Chloride anion leaves from CNBSF and GS-5NBSF is formed.
3) Subsequently, alkoxide anion of Tyr 108 attacks to sulfonyl fluoride group
4) Ternary complex is formed

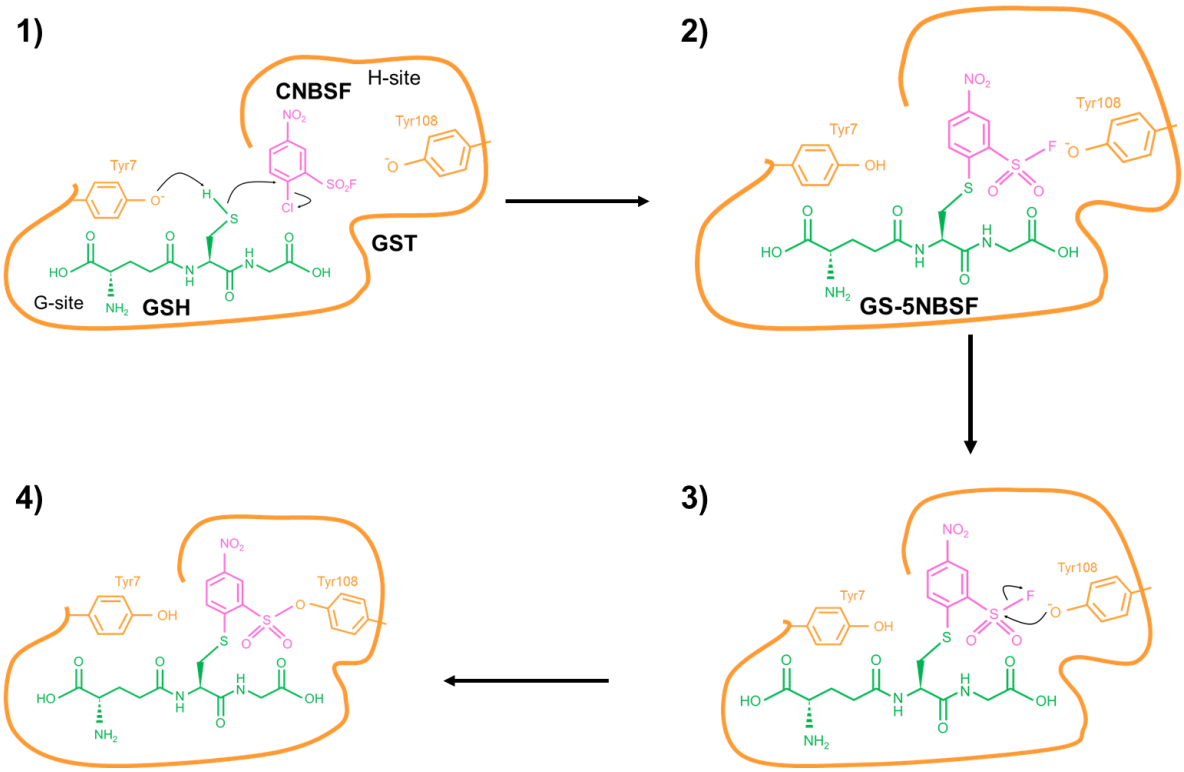


Figure A1. Putative reaction mechanism of CNBSF

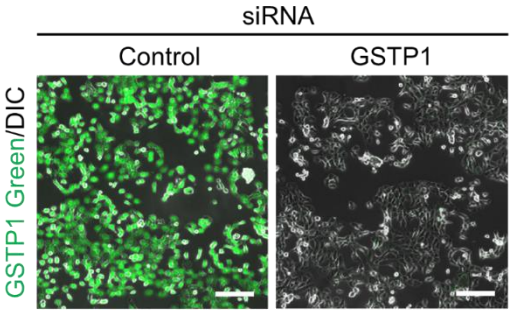
Related products

CellFluor™ GSTP1

CellFluor™ GSTP1 is an activity assay probe for pi-class GST (GSTP1). Since GSTP1 is highly expressed in various cancer cells among GST family, GSTP1 is considered as one of the anti-cancer drug-resistant and LPO-derived aldehydes neutralizing enzymes. CellFluor™ GSTP1, a world's first reagent specific for GSTP1, is only visualized GSTP1 activity among the other GST family members in live cells.

Catalog No. FDV-0034
Size 1 set
- 0.1 mg CellFluor™ GSTP1
- 0.5 mg supplemental reagent MK571)

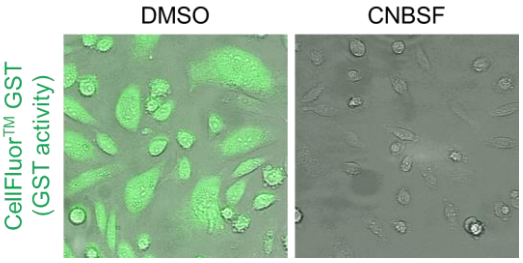
Features
- Highly specific for GSTP1 among cytosolic GST family members
- Optimized in live cell experiments (not compatible with *in vitro* assay)



CellFluor™ GST <Cell-based GST Activity Assay Reagent >

CellFluor™ GST is a novel fluorescent probe for monitoring wide GST members' activity both *in celluo* or *in vitro*. CellFluor™ GST releases green fluorophore rhodamine 110 upon GST activities. This probe has cell-permeability and can detect intracellular GST activity.

Catalog No. FDV-0030
Size 0.1 µmol
Features
- Easy and quick protocol
- Broad specificity for various GST family members
- Ex/Em: 496 nm/520 nm
  (Compatible with commercial FITC filters)



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