

Identification of Small-Molecule Frequent Hitters of Glutathione S-Transferase–Glutathione Interaction

Journal of Biomolecular Screening
1–12
© 2016 Society for Laboratory
Automation and Screening
DOI: 10.1177/1087057116639992
jbx.sagepub.com


Jara K. Brenke^{1,*}, Elena S. Salmina^{2,*†}, Larissa Ringelstetter¹,
Scarlett Dornauer¹, Maria Kuzikov³, Ina Rothenaigner¹, Kenji Schorpp¹,
Fabian Giehler^{4,5}, Jay Gopalakrishnan⁶, Arnd Kieser^{4,5}, Sheraz Gul³,
Igor V. Tetko^{7,8,†}, and Kamyar Hadian¹

Abstract

In high-throughput screening (HTS) campaigns, the binding of glutathione S-transferase (GST) to glutathione (GSH) is used for detection of GST-tagged proteins in protein-protein interactions or enzyme assays. However, many false-positives, so-called frequent hitters (FH), arise that either prevent GST/GSH interaction or interfere with assay signal generation or detection. To identify GST-FH compounds, we analyzed the data of five independent AlphaScreen-based screening campaigns to classify compounds that inhibit the GST/GSH interaction. We identified 53 compounds affecting GST/GSH binding but not influencing His-tag/Ni²⁺-NTA interaction and general AlphaScreen signals. The structures of these 53 experimentally identified GST-FHs were analyzed in chemoinformatic studies to categorize substructural features that promote interference with GST/GSH binding. Here, we confirmed several existing chemoinformatic filters and more importantly extended them as well as added novel filters that specify compounds with anti-GST/GSH activity. Selected compounds were also tested using different antibody-based GST detection technologies and exhibited no interference clearly demonstrating specificity toward their GST/GSH interaction. Thus, these newly described GST-FH will further contribute to the identification of FH compounds containing promiscuous substructures. The developed filters were uploaded to the OCHEM website (<http://ochem.eu>) and are publicly accessible for analysis of future HTS results.

Keywords

GST, glutathione, frequent hitter, AlphaScreen, high-throughput screening

Introduction

The glutathione S-transferase (GST), a 211 amino acid protein translating into a 26 kD protein, plays an important role in catalyzing the conjugation of electrophilic substrates to glutathione (GSH) for detoxification of xenobiotics.¹ In biochemical research, this protein is widely used for the production of recombinant proteins from crude bacterial lysates.² Therefore, the GST DNA sequence of the parasitic helminth *Schistosoma japonicum* is frequently integrated into different expression vectors, resulting in a fusion protein that usually carries an N-terminal GST-tag.³ Using the GST-tag for protein purification has beneficial effects as it not only promotes higher expression levels of the recombinant protein but can also act as a chaperone to improve proper protein folding. Furthermore, it enhances the expression of a soluble protein rather than protein accumulation in inclusion bodies.² In addition, the GST fusion protein can be purified without denaturation or use of mild detergents via binding to its natural substrate, the tripeptide glutathione (Glu-Cys-Gly).^{2,3}

GST fusion proteins have been successfully included in a variety of biological applications including immunological studies and vaccine production,⁴ structure determinations,^{5,6}

¹Helmholtz Zentrum München für Gesundheit und Umwelt (GmbH), Assay Development and Screening Platform, Institute of Molecular Toxicology and Pharmacology, Neuherberg, Germany

²Institute for Organic Chemistry, Technical University Bergakademie Freiberg, Germany

³Fraunhofer Institute for Molecular Biology and Applied Ecology, ScreeningPort (Fraunhofer-IME SP), Hamburg, Germany

⁴Helmholtz Zentrum München für Gesundheit und Umwelt (GmbH), Research Unit Gene Vectors, Munich, Germany

⁵German Center for Infection Research (DZIF), Partner Site Munich, Munich, Germany

⁶Laboratory for Centrosome and Cytoskeleton Biology, CMMC, Cologne, Germany

⁷Helmholtz Zentrum München für Gesundheit und Umwelt (GmbH), Institute of Structural Biology, Neuherberg, Germany

⁸BigChem GmbH, Ingolstädter Landstrasse 1, Neuherberg, Germany

*These authors contributed equally

†Chemoinformatic analysis.

Supplementary material for this article is available on the *Journal of Biomolecular Screening* Web site at <http://jbx.sagepub.com/supplemental>.

Corresponding Author:

Kamyar Hadian, Helmholtz Zentrum München GmbH, Assay Development and Screening Platform, Institute of Molecular Toxicology and Pharmacology, Ingolstädter Landstr. 1, 85764 Neuherberg, Germany.
Email: kamyar.hadian@helmholtz-muenchen.de

protein-protein and protein-DNA interaction studies, as well as other biochemical analysis.² Moreover, GST fusion proteins have been successfully employed in biochemical high-throughput screening (HTS) using the Alpha (Amplified Luminescent Proximity Homogenous Assay) technology (PerkinElmer, Waltham, MA).^{7–9} The AlphaScreen technology is a homogenous bead-based assay to study biomolecular interactions in vitro. Briefly, appropriate donor and acceptor beads bind to the corresponding tagged protein, and upon interaction of the proteins or other binding partners (e.g., peptides, DNA, RNA), singlet oxygen is transferred from the donor bead to the acceptor bead, thereby producing a luminescent signal. Compared with other technologies, AlphaScreen offers advantages as it allows a large distance of up to 200 nm between donor and acceptor beads and therefore is suitable for the use of larger proteins compared with, for example, Förster resonance energy transfer (FRET) technology, which is limited to 10 nm distance between the donor and acceptor.⁹ However, no matter which technological platform is used for conducting HTS campaigns, false-positive Hits may arise that either interfere nonspecifically with one of the assay components or with assay signal generation and/or detection. Therefore, detection of frequent hitter (FH) molecules is one of the most important steps during the hit selection process in order to progress only those compounds that are yielding genuine activity. In the AlphaScreen technology, interfering compounds might either act as singlet oxygen quenchers, color quenchers (also known as inner filters), light scatterers, or acceptor bead competitors, and the AlphaScreen TruHits Kit enables the identification of some of these artifacts. In previous studies, Baell and Holloway¹⁰ published the Pan-Assay-Interference Compounds (PAINS) filters to identify FH classes being promiscuous substructures of chromophores, redox-active, chelating, and protein reactive compounds.^{10,11} Subsequently, efforts have been made to further extend this knowledge, and chemoinformatic filters that identify FHs, which interfere with the AlphaScreen chemistry (AlphaScreen FHs) or with the His-tag/Ni²⁺-NTA interaction (His-tag FHs), have been developed and made publicly available.¹²

To establish filters that would specifically detect GST-FH compounds, we analyzed data of two different AlphaScreen HTS campaigns based on the GST/GSH interaction and three independent control screenings, with each employing 25,000 small molecules. From these studies, 53 compounds were verified as being specific GST-FHs in a confirmatory AlphaScreen assay that made use of a GST-His fusion protein. These small molecules were further analyzed in counter assays using the TruHits Kit (PerkinElmer) and a Biotin-His peptide based on AlphaScreen experiments demonstrating that these compounds are indeed specific for GST/GSH interaction. These data were the basis for developing novel chemoinformatic filters that would help to identify GST-FH in GSH-coupled matrices. Importantly, the identified GST-FH compounds did not interfere with the anti-GST antibody-based technologies, namely,

the time-resolved FRET (TR-FRET) assay and antibody-based pull-down, suggesting a difference in the bias of FHs toward GSH and anti-GST antibody-based detection of GST.

Materials and Methods

Materials

Biological Reagents. General biological reagents not listed elsewhere (e.g., buffers) were purchased from Sigma-Aldrich (St. Louis, MO) and Roth (Germany) and were of the highest available purity.

Library and Compounds. The HTS compound library comprised a diversity set of 25,000 compounds.¹² Reordered GST-FH compounds were purchased (1) from ChemDiv (San Diego, CA): GST-FH.1 (C301-6417), GST-FH.2 (M213-0587), and GST-FH.3 (M213-1079) or (2) from ENAMINE Ltd. (Princeton, NJ): GST-FH.4 (T6730144).

AlphaScreen Reagents. AlphaScreen (PerkinElmer) made use of GSH donor beads (product No. 6765300), Strept-Tactin Alpha donor beads (product No. AS106D), streptavidin donor beads (product No. 6760002), nickel chelate (Ni²⁺-NTA) donor beads (product No. AS101D), histidine (nickel chelate) detection kit (product No. 6760619C), C-Myc detection kit (product No. 6760611C), and the TruHits Kit (product No. 6760627D).

TR-FRET Reagents. TR-FRET (Cisbio, France) made use of anti-GST-XL665 (product No. 61GSTXLB) and anti-His-K (product No. 61HISKLB).

Methods

Protein Synthesis. For recombinant protein expression of the GST-His fusion protein, the DNA sequence for 6xHis-tag was introduced into the pGEX-4T-1 vector (GE Healthcare Life Sciences, Munich, Germany) and expressed in *Escherichia coli* strain BL21 RIPL upon induction using 1 mM isopropyl- β -D-thiogalactopyranoside at an OD₆₀₀ = 0.6 to 0.8. Bacterial pellets were lysed in lysis buffer containing phosphate-buffered saline (PBS) buffer pH 7.4, 0.5 mg/mL lysozyme and protease inhibitor cocktail tablet without EDTA (Roche Diagnostics, Mannheim, Germany). GST-His protein was bound to GSH sepharose 4 Fast Flow (GE Healthcare Life Sciences), followed by washing and elution of GST-His protein using 50 mM reduced GSH (Sigma-Aldrich, Taufkirchen, Germany). Desalting of the eluted protein was accomplished in PBS using HiTrap desalting columns (GE Healthcare Life Sciences) and the ÄKTA purifier system (GE Healthcare Life Sciences) to remove the remaining GSH peptides that would interfere with the assay detection technology.

Hit Selection Process. Of the data of five independent AlphaScreen HTS campaigns (two assays involving GST-tag for detection and three non-GST-containing assays), all compounds were selected that reduced the AlphaScreen signal by more than 30% in both GST-containing assays. Subsequently, these compounds were analyzed for AlphaScreen-FH and His-FH.¹² Hits positive for AlphaScreen-FH or His-FH were directly excluded. For identification of GST-FH compounds out of the hit list, the mean AlphaScreen signal values were calculated for (1) Hit Set 1 and Hit Set 2 as GST containing screens and for (2) Hit Set 3, Hit Set 4, and Hit Set 5 as non-GST-containing screens. In a first selection process, each single value of (1) needed to be lower than 70%, whereas at the same time, each single value of (2) needed to exceed 80% for the same compounds. In a second criterion, the difference of the means [(2) – (1)] needed to be higher than 30% to define the Hit as a true GST-FH compound.

AlphaScreen Assays (Primary Screening; Confirmatory and Counter Screening)

1. Screening of 25,000 compounds of five different HTS protein-protein interaction campaigns was performed using the AlphaScreen technology as previously described.¹²
2. Confirmatory AlphaScreen assays of 58 identified GST-FH compounds were accomplished using 30 nM GST-His fusion protein, GSH donor beads, and nickel-chelate acceptor beads in 10-point titration experiments.
3. For counter screening, the TruHits Kit (PerkinElmer) was conducted in 10-point titrations.¹² As an additional counter screening strategy for AlphaScreen assays, the compounds were tested in 10-point titrations and incubated with 30 nM Biotin-His peptide for 1 h and detected via streptavidin donor beads and nickel-chelate acceptor beads.

All data were processed using Excel (Microsoft Corp., Redmond, WA) and visualized using Prism (Graphpad Software, La Jolla, CA). IC₅₀ calculations were performed in ActivityBase XE (IDBS, Guildford, UK).

TR-FRET Assays. TR-FRET counter assays were performed as previously described¹² using 2 nM of GST-His protein. All data were processed using Excel (Microsoft Corp.) and visualized using Prism (Graphpad Software).

Pull-down Assays. Glutathione Sepharose 4B (GE Healthcare Life Sciences) or Ni Sepharose 6 Fast Flow (GE Healthcare Life Sciences) pull-downs were performed in assay buffer (1× PBS, 5% glycerol, 0.1% Triton-X100, protease inhibitor cocktail tablet without EDTA [Roche Diagnostics]) by preincubation of 250 ng GST-His fusion protein with the individual GST-FH compounds for 30

min. Upon addition of the respective beads (GSH or nickel) and incubation for an additional 2 h, washing was conducted six times using 1× PBS, 5% glycerol, 0.5% Triton-X100, protease inhibitor cocktail tablet without EDTA (Roche Diagnostics). Bound protein was eluted with 2× Rotiload (Roth). GST-Trap_A immunoprecipitation of the GST-His fusion protein was performed according to the protocol provided by the manufacturer (Chromotek, Planegg, Germany). Detection of the pulled GST-His protein was performed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by Western blot analysis using the GST antibody No. 2622 (Cell Signaling Technology, Beverly, MA).

Identification of Additional Experimental Data Using PubChem. For the validation of the developed substructural filters, the PubChem BioAssay database¹³ was used. A query line <AlphaScreen gst AND (pcassay_protein_target[filter]) AND (Screening[filter]) AND 1000:10000000[Total Sid Count]> retrieved only one primary screening assay implemented using AlphaScreen technology and GST-tagged protein¹⁴ (**Suppl. Table S1**). This assay identified 2589 hits and denoted this data set as “PC-Primary screen.” The database also contained several confirmatory assays. We considered compounds that were inactive in confirmatory screens as potential FHs. A query line <AlphaScreen gst AND (pcassay_protein_target[filter]) AND (Confirmatory[filter])> sifted out 19 bioassays employed GST/GSH anchoring and AlphaScreen technology (**Suppl. Table S2**). Thirteen of them had an insufficient experiment description and were not considered any further. Two assays were specifically developed to recognize artifacts of the AlphaScreen signal and incorporated dual-tagged His/GST proteins (**Suppl. Table S2**). Substances from these assays were merged into a data set named “PC-Artifacts” comprising 1266 individual compounds. Amid them there were 629 FHs (i.e., false-positive hits that decreased luminescent signal). The decrease of the luminescence in these assays can be due to one of three main reasons: interference with AlphaScreen chemistry, His-tag/Ni²⁺-NTA-specific binding,¹² or GST-tag-specific binding, which was the goal of this study. Although we could try to refine data and eliminate FHs appearing due to two first mechanisms using previously developed filters, this procedure may also potentially eliminate some GST/GSH-FHs. That is why, as with PC artifacts, we decided to use all of these data for further analysis. All of these substances were employed for validation of GST filters. The remaining four assays (**Suppl. Table S2**) comprised 1283 individual substances. These compounds were merged into the “PC-Confirmatory” data set, and likewise, “artifacts” compounds were used to evaluate the developed filters. It should be mentioned that, contrary to our previous study,¹² the PubChem bioassays were employed not only for the validation of the developed filters but also for their improvement.

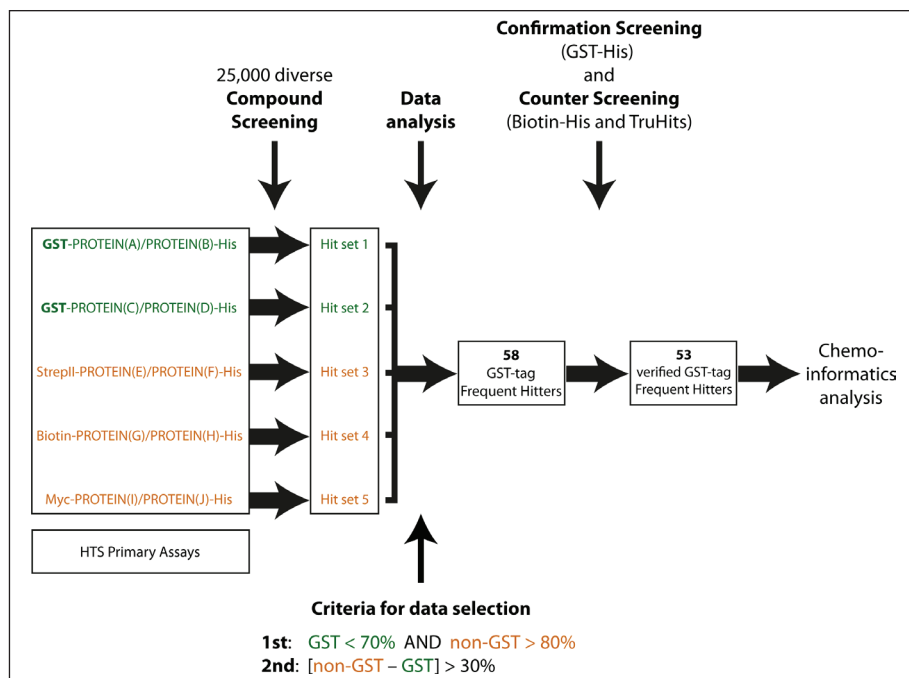


Figure 1. Overall workflow of this study. In five independent high-throughput screening campaigns comprising different protein pairs (A/B, C/D, E/F, G/H, and I/J), 25,000 compounds were tested using the AlphaScreen technology involving various detection systems: glutathione S-transferase (GST)/His (Hit set 1 and 2), StrepII/His (Hit set 3), biotin/His (Hit set 4), and myc/His (Hit set 5). For identification of GST-frequent hitter (FH) compounds, a two-step selection process was taken as a basis: (1) GST-FH had to reduce the signal to less than 70% in Hit sets 1 and 2, whereas signals of Hit sets 3, 4, and 5 needed to exceed 80%; (2) the difference of the signal means of non-GST- and GST-containing assays needed to be greater than 30%. Application of these two criteria led to 58 GST-FH. Fifty-three of these compounds were proven to be true GST-FHs by confirmatory and counter AlphaScreen assays and served as the basis for chemoinformatic analysis.

Identification of Significant Filters. For development of sub-structural filters, 53 molecules experimentally identified as GST-FHs were used. Structural analysis involved a common scaffold search, structural similarity grouping, as well as analysis of chemical activity based on the known reactive functional moieties.¹⁵ To estimate the quality of the filters, we used the assessment technique¹⁰ used in our previous publication.¹² Considering that we had a smaller number of assays compared with both previous studies, we calculated the enrichment value (EV_{GST}) with the following equation:

$$EV_{GST} = \frac{HITS_{2/0} + HITS_{2/1} + HITS_{1/0}}{\sum_{j=0}^3 HITS_{0/j}},$$

where $HITS_{i/j}$ shows the number of compounds that were active in the i GST/GSH assays ($i = 0, 1, 2$) and j GST/GSH-free assays ($j = 0, 1, 2, 3$) simultaneously. Filters with $EV_{GST} \geq 30\%$ (i.e., the same criterion as used previously^{10,12}) were considered for inclusion in the set of GST-FH (GST-FH-S).

Results and Discussion

Detection and Confirmation of GST-FH Compounds from Multiple AlphaScreen HTS Campaigns

To identify GST-FH compounds that interfere with the GST and GSH binding, the data of five previous AlphaScreen-based

HTS campaigns, each exploiting different target protein-protein interactions (**Fig. 1**), were used to perform an in-depth analysis. These screens included two assays, both of which involved the GST/His-tag combination (Hit set 1 and 2). The GSH donor beads bound the GST-tagged protein, and the His-protein was detected via nickel-chelate acceptor beads. The control screens used the combinations of StrepII/His-tag (Hit set 3), biotin/His-tag (Hit set 4), and myc/His-tag (Hit set 5; **Fig. 1**). For identification of GST-FHs within the 25,000 tested small molecules for each assay combination, data were analyzed as follows: In a first selection step, GST-FH had to reduce the AlphaScreen signal by more than 30% in both GST-containing assays, whereas, at the same time, the signals for all non-GST-containing assays needed to be higher than 80% (i.e., GST < 70% and non-GST > 80% AlphaScreen signal). The second step included a criterion for GST-FHs when the difference of the mean AlphaScreen signal of all non-GST-containing assays and all GST-containing assays exceeded 30% (i.e., non-GST - GST > 30%; **Fig. 1**). Importantly, for flagging the respective compound as a GST-FH, both criteria needed to be fulfilled at the same time. Of 25,000 compounds, we were able to extract 58 small molecules that achieved our predefined two-step criteria. Notably, these were not overlapping with compounds that were already classified as general FHs of the AlphaScreen technology or His-FH interfering with the His-tag/ Ni^{2+} -NTA interaction.¹²

Subsequently, these 58 compounds were tested in 10-point titration in AlphaScreen assays starting from 50 μ M as the highest compound concentration (**Fig. 2A**). Here, the small molecules were examined using a recombinantly expressed

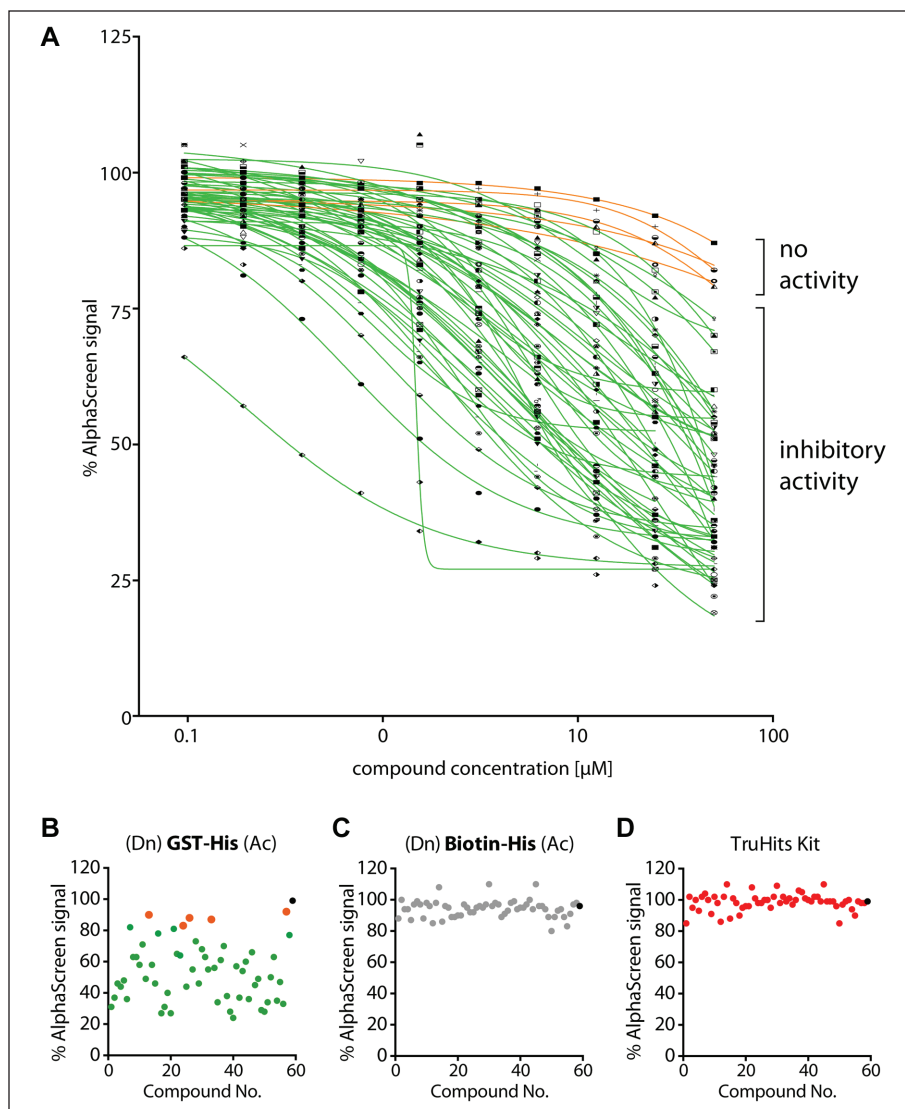


Figure 2. Analysis of glutathione S-transferase (GST)-frequent hitter in confirmatory and counter AlphaScreen assays. **(A)** The activity of 58 compounds was tested in 10-point titration experiments. Fifty-three compounds displayed a decent inhibitory activity (green), whereas five compounds exhibited no activity (orange) **(B–D)**. All compounds were tested at a concentration of 25 μ M using **(B)** GST-His fusion protein (most compounds had decent inhibitory activity on the AlphaScreen signal), **(C)** biotin-His peptide control AlphaScreen assay (none of the tested compounds interfered with this assay), or **(D)** the TruHits control assay (none of the tested compounds interfered with this assay). Dn, donor; Ac, acceptor. DMSO control is labeled in black.

GST-His fusion protein, consisting of the GST protein with a C-terminal His-tag that was detected via GSH donor and nickel-chelate acceptor beads. In total, 5 of the 58 tested compounds did not affect the GST-His assay (**Fig. 2A**, orange label), and 53 compounds indeed impaired the GST-His AlphaScreen signal with decent activity at least at the maximum concentration of 50 μ M (**Fig. 2A**, green label). For 37 of 53 active compounds, IC_{50} values could be calculated, and these values are depicted in **Table 1**. Furthermore, biotin-His peptide (streptavidin donor beads and nickel-chelate acceptor beads) as well as the TruHits Kit (streptavidin donor beads and biotinylated acceptor beads) served as controls that should not be affected by the compounds. Although most of the compounds reduced the AlphaScreen signal in the GST-His assay at 25 μ M compound concentration (**Fig. 2B**), all 58 tested compounds did not interfere with the biotin-His peptide assay nor affected the TruHits assay (**Fig. 2C, D**). This is in fact very

important as it verifies that these compounds do not interfere with the AlphaScreen technology itself, nor do they impair the His-tag/ Ni^{2+} -NTA binding.

Thus, we provide an experimental proof that 53 compounds certainly interfered with the GST/GSH interaction with different levels of severity and can herewith be classified as GST-FHs. These compounds were then subjected to a chemoinformatic analysis to discover substructures that are responsible for disrupting the GST/GSH interaction.

Preliminary Data Analysis

In the preliminary analysis, 53 GST-FHs were screened against existing substructural filters recognizing promiscuous¹⁶ and PAINS compounds¹⁰ using the ToxAlerts tool¹⁷ of the OCHEM (www.ochem.eu) platform.¹⁸ Eight compounds were recognized as PAINS and three as promiscuous compounds (**Suppl.**

Table 1. IC₅₀ values of glutathione S-transferase (GST)–frequent hitter.

OCHEM ID ^a	IC ₅₀ (μM)	OCHEM ID	IC ₅₀ (μM)	OCHEM ID	IC ₅₀ (μM)
M1108012	44.9	M1220470	17.77	M1024891	7.3
M1108832	43.45	M1109005	16.77	M2682879	7.19
M1108852	40.18	M2989302	15.84	M1213554	6.79
M1108059	38.97	M1040098	14.39	M3383345	6.68
M1108836	37.47	M1113194	10.84	M1107602	6.64
M1108964	36.48	M771355	9.79	M3200831 (GST-FH.3)	5.01
M2679239	30.72	M1024342	9.37	M1114268	4.43
M1215709	25.91	M1111647	9.09	M1217802 (GST-FH.2)	3.87
M1231217 (GST-FH.4)	24.38	M1110421	8.8	M2746788	2.86
M416273	24.16	M306932	8.68	M1226684	1.65
M1230853	23.03	M1232295	8.36	M2728825	1.27
M1024320	22.02	M1040939	8.14	M1111171 (GST-FH.1)	0.32
M1113931	20.03				

^aThe IC₅₀ values and molecular structures are freely available for download at <http://ochem.eu/article/93878>.

Table S3, S4, respectively). This result was expected, because two out of five high-throughput screening assays (all AlphaScreen technology) employed for developing PAINS filters incorporated GST-tagged proteins, thus contributing to the sensitivity of PAINS filters to GST-FHs. Therefore, both of these studies were valuable sources of filters for our study. Below, we analyzed and extended some of these filters to provide higher coverage of FHs analyzed in this study.

Extension of PAINS Filters

- Two GST-FHs among PAINS-recognized compounds were identified by the “Dhp_bis_amino_CN” filter developed for aminocyanothiopyrans,¹⁰ and this filter was reported to have a very high EV (1400%). Screening of our data has revealed that this filter was also highly specific to GST-FHs (EV = 230%). Because none of the analyzed compounds contained oxa- or aza-analogues of the thiopyran core, we added the corresponding PAINS filter to GST-FH-S without any modifications. One compound with a structurally similar dithione group was also found among GST-FHs. However, because there were no other compounds with this group present either in the Helmholtz Zentrum München (HMGU) or in the PubChem data sets, it was not possible to estimate reliably the contribution of the variance of substituents within this chemical class to its false activity in GST/GSH–employing bioassays (further, we would refer to it as *GST promiscuity*). Therefore, we created an individual singleton FH filter for this compound.
- A “Keto_phenone_A” PAINS filter for 9-fluorenone azaderivatives recognized one GST-FH out of three compounds with this structural moiety. This filter was extended to cover all three compounds (**Suppl. Fig. S1**) with $EV_{GST} = 57\%$.
- Promiscuous *o*-cyanothiopyridinones were correctly recognized as FHs by the “Cyano_pyridone_A” PAINS filter, which was added to GST-FH-S without modification. The *p*-cyanopyridinone moiety was also presented as a part of the fused cyclic system for one GST-FH, and this compound provided more than 50% signal reduction in GST-involving AlphaScreen assays and no signal reduction in GST-free assays. However, there were 16 highly structurally similar *p*-cyanopyridinones among substances that were inactive in GST/GSH-containing bioassays (further referred to as “clean” substances). Thus, a selective filter to identify this FH was created. Another GST-FH containing a pyridinone moiety with an electron-withdrawing substituent was recognized by the PAINS filter “Het_65_E.” One additional GST-FH with a pyridinone fragment was found among GST-FHs. We were not able to extend any of the aforementioned filters for the (thio)pyridinones, and a separate filter was created for this singleton (see “Pyranopyridinones” filter in **Suppl. Table S5**).
- A PAINS filter “Quinone_A” recognized the GST-FH-containing anthraquinone scaffold. Quinones are well-known electrophilic agents containing an activated double bond, which is capable of binding covalently to nucleophilic moieties. Accordingly, it can be assumed that they bind to the thiol group of the GSH-modifying GST substrate and prevent anchoring of tagged proteins. However, in anthraquinones, the quinoid structure is a part of the aromatic system and does not behave as an activated double bond. Analysis showed that 24 molecules

comprised anthraquinone-like structures, which did not provoke any decay in their fluorescent signals. In our opinion, the GST promiscuity of this FH is not solely governed by the presence of a quinone moiety (section “Remarks on the Filters”). Therefore, the corresponding PAINS filter was not incorporated into our GST-FH-S. Instead, a singleton-like filter was created to cover this FH. It can be further extended once more diverse structural information appears.

5. A PAINS filter “Ene_five_het_H” identifies thioazolones with exocyclic double bonds (hidden Michael acceptors). Among the studied GST-FHs, an oxo-analogue also appeared. The filter was augmented to cover the latter and was saved under the shared name “Pyrazolones.”
6. A PAINS filter “Catechol_A” recognized the only GST with a catechol fragment but showed a rather low EV value (17%). We modified the filter by including the molecular scaffold to which the catechol ring belongs (section “Remarks on the Filters”).
7. Although the PAINS filter “Thio_carbonate_A” has not recognized GST-FHs by itself, it describes a molecular fragment that is highly structurally similar to a GST-promiscuous benzoxathiolone. After a slight modification, the filter was added to GST-FH-S as well.

The aforementioned PAINS filters and/or their extensions (seven in total) were included in GST-FH-S. Although some of these filters were simply duplicates of PAINS filters, their inclusion in the GST-FH collection could provide an important hypothesis to the users about the mode of action of FH and can guide them to design counter screens to validate the hits.

8. The “artifacts” data set contained many aromatic sulfonamides having common fragments depicted in **Fig. 3A** (see also **Suppl. Table S6**). Compounds with the same moiety were also present among GST-FHs. However, this fragment alone did not distinguish between GST-FHs and “clean” substances. PAINS filters contained more elaborated aromatic sulfonamide filters (“Diazox_sulfon_A” and “Diazox_sulfon_B”), which covered compounds structurally similar to GST-FH aromatic sulfonamides. Both PAINS filters were extended to account for this type of GST-FHs (section “Remarks on the Filters”).

The filters for promiscuous substances¹⁶ shifted out three GST-FHs (**Suppl. Table S4**). One of these compounds was also recognized by PAINS filters and thus was not further analyzed. The second compound was the only thiopyrylium-comprising substance in both HMGU and PubChem data

sets. This did not allow us to explore the structural variability around the thiopyrylium scaffold. The corresponding promiscuous substances filter was added to our GST-FH-S collection without any modification (**Suppl. Table S5**). GST-FH identified by the third filter (named “Linear_polycyclic_aromatic_I”¹⁶) contained a pyrimidinone substructure. This compound was recognized by a filter developed for pyrimidinones, as explained later in this article.

Analysis of FH Not Covered with Existing FH Filters

After applying 14 aforementioned filters, 35 GST-FHs remained undetected. For development of the new filters, we assumed that the GST promiscuity of the studied substances could be due to one of the following reasons:

1. Occupation of GSH binding site of GST (G-site) due to structural similarity between the GSH substrate and studied substances. This mechanism can be identified by a search of ligands structurally resembling GSH.
2. Irreversible conjugation of a molecule with Cys-residues of immobilized GSH leading to the inability of recombinant proteins to anchor to bead surfaces. This mechanism applies to reactive species with sufficiently strong electrophilic properties.
3. Reversible hydrogen bonding of the molecule to sites important for GST-GSH interaction. This mechanism applies to substances with strong hydrogen bond donating/accepting moieties.

Analysis of structural similarity showed that there were no molecules structurally similar (Tanimoto <60%) to GSH among the studied GST-FHs, thus eliminating the first hypothesis.

To identify reactive species with prominent electrophilic features, we used filters to recognize potential electrophilic agents (PEAs)¹⁹ available in ToxAlerts.¹⁷ Application of PEA filters showed that 7 of 35 remaining GST-FHs comprised electrophilic moieties (**Suppl. Table S7**). Unfortunately, PEA filters had low specificity (**Suppl. Table S8**) and did not pass EV quality criteria to be included in the list of the GST-FH filter set. Indeed, these filters were developed for recognition of protein-reactive agents. Therefore, they inherently accounted for precursors of such molecules, which are metabolically bio-transformed to gain their chemical activity. For example, the PEA filter “hydroquinones” recognized four GST-FHs containing the alkoxybenzene fragment as well as 510 compounds that were not FHs. In vivo alkoxybenzenes are readily converted into hydroquinones by methyltransferases with subsequent oxidation into reactive quinones.²⁰ The experimental conditions of the studied assays did not provide the

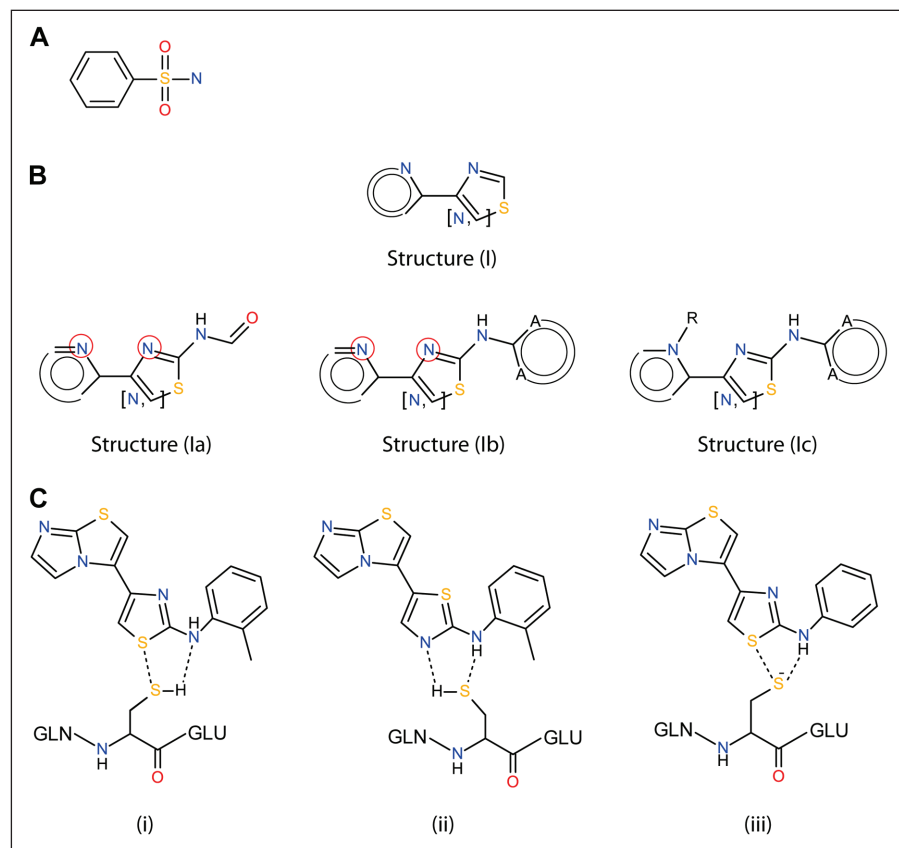


Figure 3. Chemoinformatic analysis. **(A)** Common moiety for aromatic sulfonamides presented in the “artifacts” data set. **(B)** 4-Heteryl-substituted thiazoles frequently observed among promiscuous compounds in the Helmholtz Zentrum München and PubChem data sets (R = any atom/group). The Ic subtype is specific for glutathione S-transferase (GST)-frequent hitter (FH). **(C)** Proposed mechanism of action of GST-FHs containing the arylaminothiazoles moiety. The hypothetical specific interactions are shown by the dashed lines.

opportunity for such chemical transformation by contributing a large number of compounds that were not FHs in our study and provided EV values less than 4%. Thus, despite the high plausibility of the second hypothesis, we could not use these filters in our analysis. For some of these compounds, we developed singleton-like filters, as discussed below.

Analysis of FH Based on Hydrogen-Bond Formation

The potential to form hydrogen bonds with the receptor was analyzed as the last hypothesis to provide a mechanistic-based development of FHs.

Among the GST-FHs, the most prevalent compounds were 4-heteryl substituted thiazoles comprising the common structure (I) depicted in **Fig. 3B**. Compounds containing this structure were frequently found among the artifacts of AlphaScreen technology as well as among active compounds in the primary screen assay. Detailed structural examination divided them into three main subgroups containing common substructures (**Fig. 3B, Ia–c**). Although confirmed GST-FHs contained only substructure (Ic), active PubChem compounds incorporated either substructure (Ia) or (Ib). It was also observed that chemicals from the HMGU collection comprising substructure (Ia) were frequently active in all five studied assays. The behavior

of chemicals from subgroups (Ia) and (Ib) can be explained by the presence of the $\text{N}_{sp^2}\text{CCN}_{sp^2}$ group (marked by red circles in **Fig. 3B**) in their structure. According to the results of our previous study, these compounds can act as chelating agents to Ni^{2+} -ions/His-residues, causing a false decay of the luminescent signal. Because all considered assays incorporated a His/ Ni^{2+} -anchoring system, a false activity of these compounds most likely flows from chelating abilities and is not related to the GST/GSH system. Importantly, in GST-FHs of this class, the substructure $\text{N}_{sp^2}\text{CCN}_{sp^2}$ is replaced by the $\text{N}_{sp^2}\text{CCN}_{sp^3}$ fragment (**Fig. 3B, Ic**), which causes a distraction of a chelating center and subsequent loss of chelating properties of compounds toward His-Tag/ Ni^{2+} -NTA. In fact, two GST-FHs also contain the $\text{N}_{sp^2}\text{CCN}_{sp^2}$ fragment, where one N_{sp^2} is a part of triazole ring. It is well known that due to the presence of three electronegative nitrogens in the triazole ring, these molecules are very weak bases. Therefore, the lone pairs of the nitrogen atoms normally do not participate in complex formation.²¹

The GST promiscuity of this type of GST-FHs is provoked by specific noncovalent interactions between FHs and thiol groups of immobilized GSH rather than covalent binding of this group with proteins. We came to this assumption after structural comparison of GST-FHs and “clean” compounds. Indeed, substituent in the fourth position of a

thiazole ring likely does not play any role on GST promiscuity because it varies within GST-FHs. A fragment, which is presented in all FHs, includes thiazolyl-substituted aromatic secondary amine (**Fig. 3B**, Ic), and it can form non-covalent bonds with the Cys thiol-group. The Cys thiol-group has a $pK_a = 9.1$,²² and in the experiment condition ($pH = 7.4$), the neutral form should be dominated. Thus, the neutral form could participate in hydrogen bond formation, as proposed in **Figure 3C/i** and **Figure 3C/ii** (potential interactions are shown by the dashed lines).

The first hypothetical mechanism assumes formation of an H-N $\cdots$ H-S hydrogen bond and S $\cdots$ S interactions. The latter are widely distributed in proteins and crystals.^{23,24} The bond H-N $\cdots$ H-S is formed by secondary amine nitrogen with two aromatic substituents (**Fig. 3C/i**). H-donating properties of this type of the nitrogen atom are decreased due to a steric hindrance of its electron pair, and so this type of interaction may not be sufficiently strong to form the complexes.

The second proposed mechanism is based on N $\cdots$ H-S and S $\cdots$ H-N interactions, which are also observed in crystals of proteins and organic molecules.^{25–29} However, because of the weak hydrogen bond accepting properties of the S atom in the Cys group, an S $\cdots$ H-N bond is likely to be rather weak. Thus, this hypothesis is also not very likely.

The third hypothesis is based on the assumption that, despite that the pK_a value is higher than that of the experiment value, the Cys thiol group can be still ionized. It is known that a local microenvironment created by the presence of a large number of polar groups on bead surfaces (i.e., thiol, carboxyl, and amino groups) can shift the pK_a of the Cys thiol group, increasing the concentration of thiolate form.^{30–32} Hence, the lone electron pair of thiolate-S can participate in an anion hydrogen bond with acidic hydrogen of the amino group, whereas core sulfur electrons form donor-acceptor S $\cdots$ S-specific interactions (**Fig. 3C/iii**). This hypothesis looks the most convincing for us, because anion-hydrogen bonds are strong interactions and, being additionally fortified by specific S $\cdots$ S contacts, should form a stable complex.

The aforementioned three hypotheses are based on the observations that about 60% of the studied GST-FHs contain thio-functionalities in their structure. Indeed, in some cases, corresponding oxo-analogues of the studied hitters do not interfere with GST/GSH-involving assays (**Suppl. Table S9, S10**). It might be due to a specific role of S $\cdots$ S interactions in the GST promiscuity of the studied FHs.

Verification of GST-FH Compounds in TR-FRET and Pull-Down Assays

For further investigation, four compounds were selected using the following criteria: (1) specific inhibition of the

GST/GSH interaction, (2) IC_{50} below 25 μM (**Fig. 2A**; **Table 1**), and (3) belonging to different classes of small-molecule families: GST-FH.1 (M1111171) belonging to the family of the piperazinediones, GST-FH.2 (M1217802) and GST-FH.3 (M3200831) representing the 4-heteryl-thiazoles family, as well as GST-FH.4 (M1231217) as a member of the sulfonamide_A group (**Fig. 4A**). All four GST-FH were tested in a dose-response study on the GST-His AlphaScreen assay, thereby exhibiting decent inhibition of the AlphaScreen luminescence signal (**Fig. 4B**). In contrast, when adding the GST-FH compounds in the same concentration range to the biotin-His peptide, production of a stable AlphaScreen signal was achieved that was completely unaffected (**Fig. 4C**). This dose-response verification again underlined that the tested compounds are GST-FHs rather than general AlphaScreen or His-FH compounds.

To verify the interference of the GST-FH compounds with the GST/GSH interaction, TR-FRET assays that detect the GST-His fusion protein via an anti-GST antibody rather than by GSH-coupled matrix were performed. Therefore, the GST-His fusion protein was incubated with the respective GST-FH compound up to 100 μM and detected via a MAb anti-GST-XL665 (acceptor) and a Mab anti-His-K (donor). In contrast to the AlphaScreen experiments, even at a concentration of 100 μM , all four analyzed compounds did not impair the binding of the anti-GST antibody to the GST-tag (**Fig. 4D**), suggesting a different binding mode of GSH donor beads and anti-GST antibody to the GST-tag. Of note, GST-FH.1 and GST-FH.2 appear to be slightly autofluorescent at the wavelength of 615 nm at high concentrations (**Fig. 4D**).

For further verification, compound GST-FH.1 with the most pronounced inhibition activity on GST/GSH binding in AlphaScreen was selected to prove its inhibitory impact on the GST/GSH interaction in pull-down assays, a very prominent assay type for analyzing protein-protein interactions in low-throughput. Importantly, this assay technology is very distinct from the AlphaScreen technology. To this end, the GST-His fusion protein was incubated with the GST-FH.1 compound and subsequently extracted using GSH-sepharose beads, GST-Traps (antibody-based), or nickel-sepharose beads. The latter two matrices served as controls. Pull-down of GST-His was dose-dependently reduced when applying GST-FH.1 to a GSH-sepharose-based pull-down assay (**Fig. 4E**). Differences in the potency of GST-FH.1 between AlphaScreen and pull-down experiment are due to different amounts of protein and bead input as well as the distinct nature of the assay technologies. In contrast, pull-down of the GST-His by either antibody-based GST-Trap or nickel-sepharose beads was not impaired by GST-FH.1, again proving that this small molecule specifically inhibits the GST-GSH interaction (**Fig. 4E**).

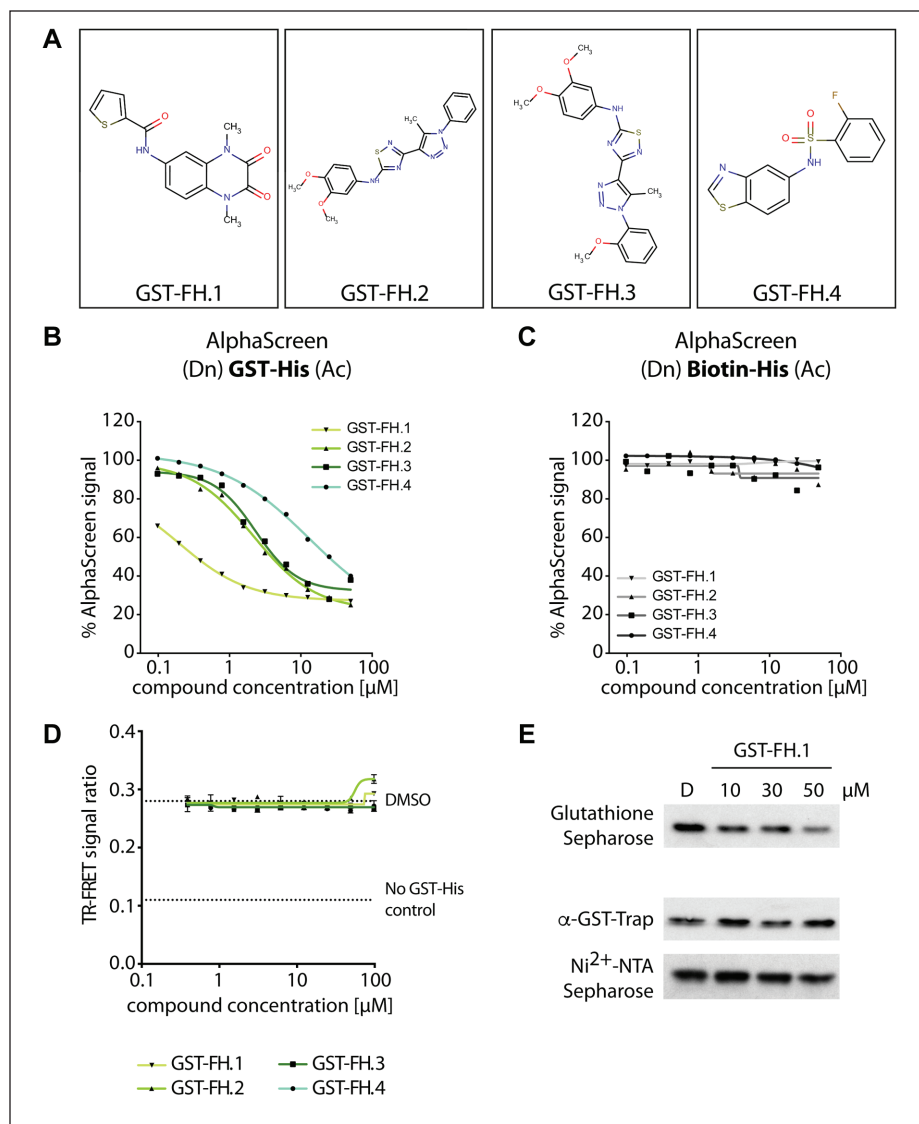


Figure 4. Analysis of selected compounds in the AlphaScreen assays, time-resolved Förster resonance energy transfer (TR-FRET), and pull-down assays. **(A)** Molecular structures of four identified glutathione S-transferase frequent hitter (GST-FH) compounds representing the family of the piperazinediones (GST-FH.1), the 4-heteryl-thiazoles (GST-FH.2 and GST-FH.3), and the sulfonamide_A (GST-FH.4). **(B)** The four selected GST-FH compounds showed dose-dependent effects in reducing AlphaScreen signals generated from a GST-His fusion protein. **(C)** In contrast, the biotin-His peptide AlphaScreen assay remained unaffected by the four compounds. **(D)** TR-FRET assays detecting the GST-His protein by antibodies were not impaired by the four selected compounds. **(E)** The most active compound, GST-FH.1, was tested in pull-down assays by extracting the GST-His protein via glutathione sepharose, GST-Trap (antibody based), or Ni²⁺-NTA sepharose matrices. Thereby, only the glutathione sepharose pull-down was dose-dependently impaired. α-GST-antibody and Ni²⁺-NTA sepharose assays were not affected. Dn, donor; Ac, acceptor.

In HTS campaigns, proteins or enzymes are often tagged to have the ability to use different matrices for output detection. Here, various tags such as His, StrepII, or GST are commonly used. The major problem with these tags is that many small-molecule compounds affect the binding of the tag to the corresponding matrix, thereby generating false-positive signals.¹² These compounds are considered to be FHs.^{10–12} In this study, we wanted to identify and describe novel cheminformatic filters that detect FHs of the GST/GSH interaction.

In total, 13 GST-FHs contained pyrimidinone fragments. The sensitivity of these compounds to the GST/GSH system was strongly affected by the core structure as well as its substituents. We clustered these compounds into six groups sharing a common scaffold and developed corresponding filters for each cluster (Suppl. Table S5).

These and other previously developed filters covered 37 of 53 detected GST-FHs. The remaining 17 compounds showed high diversity. For some of them, even small structural changes resulted in alteration of their promiscuity toward the GST/GSH system. For example, for benzopiperazinediones, a substitution of a benzene hydrogen by methyl group resulted into transformation of GST-FH into a “clean” compound (Suppl. Table S11). Because we could not provide a mechanistic interpretation of such behavior, we created a highly specific filter to cover this functional class. A similar approach was used for the remaining GST-FHs. These highly specific filters could be used as a starting point for further analysis, once new experimental data are available. In total, 34 GST-FH filters were developed and deposited and are freely available from the ToxAlerts database.¹⁷

Although chemoinformatic filters are very valuable tools for excluding FH compounds, counter assays will always be required to experimentally verify genuine Hit compounds from the list of primary Hits. Ideally, counter screening assays should be based on technologies different from the primary screening assay or need to include appropriate control proteins or reagents. For conducting an HTS involving a GST-tagged protein, one can choose between anti-GST antibody-coated agents or GSH-coated matrix. Here, the affinity of GSH for binding GST ($K_d \approx 5 \mu\text{M}$) is weaker compared with anti-GST antibodies.³³ Therefore, GST-FH small molecules might be more efficient in impairing the GST/GSH interaction in comparison with GST/anti-GST antibody binding. Another point to consider is that GSH and anti-GST antibodies most probably bind at distinct binding sites of the GST protein. Thereby, a small molecule might block the GSH-binding pocket, whereas the anti-GST antibody binding site will be still free for interaction.

Altogether, this study has developed highly important GST-FH filters that allow HTS researchers to pinpoint promiscuous GST-tag protein-specific Hits in the primary Hit list. These potential promiscuous compounds could be further tested with another technology or another tag protein to validate the Hits and exclude nonspecific ones. Future studies will help to extent and/or fine-tune these filters in order to optimize their coverage and specificity.

Acknowledgments

We also thank ChemAxon (<http://www.chemaxon.com>) for contributing their software tools used in this study.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was partially supported by the FP7 Marie Curie Initial Training Network project Environmental ChemOinformatics (ECO), grant agreement 238701, as well as by the German Research Center for Infection Research (DZIF), TTU 07.802.

References

1. Sheehan, D.; Meade, G.; Foley, V. M.; et al. Structure, Function and Evolution of Glutathione Transferases: Implications for Classification of Non-Mammalian Members of an Ancient Enzyme Superfamily. *Biochem. J.* **2001**, *360*(Pt 1), 1–16.
2. Harper, S.; Speicher, D. W. Purification of Proteins Fused to Glutathione S-Transferase. *Methods Mol. Biol.* **2011**, *681*, 259–280.
3. Smith, D. B.; Johnson, K. S. Single-Step Purification of Polypeptides Expressed in *Escherichia coli* as Fusions with Glutathione S-Transferase. *Gene* **1988**, *67*, 31–40.
4. Yip, Y. L.; Smith, G.; Ward, R. L. Comparison of Phage pIII, pVIII and GST as Carrier Proteins for Peptide Immunisation in Balb/c Mice. *Immunol. Lett.* **2001**, *79*, 197–202.
5. Zhan, Y.; Song, X.; Zhou, G. W. Structural Analysis of Regulatory Protein Domains Using GST-Fusion Proteins. *Gene* **2001**, *281*, 1–9.
6. Smyth, D. R.; Mrozkiewicz, M. K.; McGrath, W. J.; et al. Crystal Structures of Fusion Proteins with Large-Affinity Tags. *Protein. Sci.* **2003**, *12*, 1313–1322.
7. Ungermannova, D.; Lee, J.; Zhang, G.; et al. High-Throughput Screening AlphaScreen Assay for Identification of Small-Molecule Inhibitors of Ubiquitin E3 Ligase SCFSkp2-Cks1. *J. Biomol. Screen.* **2013**, *18*, 910–920.
8. Tang, X.; Seyb, K. I.; Huang, M.; et al. A High-Throughput Screening Method for Small-Molecule Inhibitors of the Aberrant Mutant SOD1 and Dynein Complex Interaction. *J. Biomol. Screen.* **2012**, *17*, 314–326.
9. Gul, S.; Hadian, K. Protein-Protein Interaction Modulator Drug Discovery: Past Efforts and Future Opportunities Using a Rich Source of Low- and High-Throughput Screening Assays. *Expert. Opin. Drug. Discov.* **2014**, *9*, 1393–1404.
10. Baell, J. B.; Holloway, G. A. New Substructure Filters for Removal of Pan Assay Interference Compounds (PAINS) from Screening Libraries and for Their Exclusion in Bioassays. *J. Med. Chem.* **2010**, *53*, 2719–2740.
11. Baell, J.; Walters, M. A. Chemistry: Chemical Con Artists Foil Drug Discovery. *Nature* **2014**, *513*, 481–483.
12. Schorpp, K.; Rothenaigner, I.; Salmina, E.; et al. Identification of Small-Molecule Frequent Hitters from AlphaScreen High-Throughput Screens. *J. Biomol. Screen.* **2014**, *19*, 715–726.
13. Wang, Y.; Xiao, J.; Suzek, T. O.; et al. PubChem's BioAssay Database. *Nucleic. Acids Res.* **2012**, *40*(Database issue), D400–D412.
14. PubChem. National Center for Biotechnology Information. PubChem BioAssay Database, AID=623870, Source=Scripps Research Institute Molecular Screening Center. <http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=623870>.
15. Salmina, E. S.; Haider, N.; Tetko, I. V. Extended Functional Groups (EFG): An Efficient Set for Chemical Characterization and Structure-Activity Relationship Studies of Chemical Compounds. *Molecules* **2016**, *21* (1).
16. Pearce, B. C.; Sofia, M. J.; Good, A. C.; et al. An Empirical Process for the Design of High-Throughput Screening Deck Filters. *J. Chem. Inf. Model* **2006**, *46*, 1060–1068.
17. Sushko, I.; Salmina, E.; Potemkin, V. A.; et al. ToxAlerts: A Web Server of Structural Alerts for Toxic Chemicals and Compounds with Potential Adverse Reactions. *J. Chem. Inf. Model* **2012**, *52*, 2310–2316.
18. Sushko, I.; Novotarskyi, S.; Korner, R.; et al. Online Chemical Modeling Environment (OCHEM): Web Platform for Data Storage, Model Development and Publishing of Chemical Information. *J. Comput. Aided Mol. Des.* **2011**, *25*, 533–554.
19. Enoch, S. J.; Ellison, C. M.; Schultz, T. W.; et al. A Review of the Electrophilic Reaction Chemistry Involved in Covalent Protein Binding Relevant to Toxicity. *Crit. Rev. Toxicol.* **2011**, *41*, 783–802.

20. Gerner, I.; Barratt, M. D.; Zinke, S.; et al. Development and Prevalidation of a List of Structure-Activity Relationship Rules to Be Used in Expert Systems for Prediction of the Skin-Sensitising Properties of Chemicals. *Altern. Lab. Anim.* **2004**, *32*, 487–509.
21. Katritzky, A. R.; Karelson, M.; Malhotra, N. Heterocyclic Aromaticity. *Heterocycles* **1991**, *32*, 127–161.
22. Tang, S. S.; Chang, G. G. Kinetic Characterization of the Endogenous Glutathione Transferase Activity of Octopus Lens S-Crystallin. *J. Biochem.* **1996**, *119*, 1182–1188.
23. Iwaoka, M.; Isozumi, N. Hypervalent Nonbonded Interactions of a Divalent Sulfur Atom. Implications in Protein Architecture and the Functions. *Molecules* **2012**, *17*, 7266–7283.
24. Desiraju, G. R.; Nalini, V. Database Analysis of Crystal-Structure-Determining Interactions Involving Sulphur: Implications for the Design of Organic Metals. *J. Mater. Chem.* **1991**, *1*, 201–203.
25. Rose, G. D.; Gierasch, L. M.; Smith, J. A. Turns in Peptides and Proteins. *Adv. Protein. Chem.* **1985**, *37*, 1–109.
26. Beheshti, A.; Clegg, W.; Dale, S. H.; et al. N-H...S and N-H...O Hydrogen Bonding in Tetra-n-Butylammonium (Imidazolidine-2-thione-2[kappa]S)dioxo-1[kappa]2O-di-[mu]-sulfido-1:2[kappa]4S:S-copper(I)tungstate(VI). *Acta. Crystallogr. Sect. E* **2005**, *61*, m1509–m1511.
27. Hutzler, W. M.; Egert, E. N-H...S and N-H...O Hydrogen Bonds: 'Pure' and 'Mixed' R22(8) Patterns in the Crystal Structures of Eight 2-Thiouracil Derivatives. *Acta. Crystallogr. Sect. C* **2014**, *70*, 241–249.
28. Scheiner, S. *Noncovalent Forces*; Springer: New York, **2015**.
29. Desiraju, G. R.; Steiner, T. *The Weak Hydrogen Bond in Structural Chemistry and Biology*; Oxford University Press: Oxford, **2001**.
30. Wang, P. F.; McLeish, M. J.; Kneen, M. M.; et al. An Unusually Low pK(a) for Cys282 in the Active Site of Human Muscle Creatine Kinase. *Biochemistry* **2001**, *40*, 11698–11705.
31. Harris, T. K.; Turner, G. J. Structural Basis of Perturbed pKa Values of Catalytic Groups in Enzyme Active Sites. *IUBMB Life* **2002**, *53*(2), 85–98.
32. Copley, S. D.; Novak, W. R.; Babbitt, P. C. Divergence of Function in the Thioredoxin Fold Suprafamily: Evidence for Evolution of Peroxiredoxins from a Thioredoxin-Like Ancestor. *Biochemistry* **2004**, *43*, 13981–13995.
33. Tessema, M.; Simons, P. C.; Cimino, D. F.; et al. Glutathione-S-Transferase-Green Fluorescent Protein Fusion Protein Reveals Slow Dissociation from High Site Density Beads and Measures Free GSH. *Cytometry A* **2006**, *69*, 326–334.