Title: Selenoprotein synthesis is not induced by hepatotoxic drugs

Authors Names: Sumangala P. Shetty, Dongming He, Paul R. Copeland*

Department of Biochemistry and Molecular Biology, Rutgers - Robert Wood Johnson Medical School, Piscataway, NJ USA

***Correspondence:**

Paul R. Copeland

Department of Biochemistry and Molecular Biology

Rutgers - Robert Wood Johnson Medical School

675 Hoes Ln, Piscataway, NJ 08854 USA

E-mail address: paul.copeland@rutgers.edu

Highlights:

- The treatment of liver cells with hepatotoxic and hepatotropic compounds does not result in increased synthesis of selenoproteins.
- Compounds that induced the canonical oxidative stress response that features NRF2 activation eliminated selenoprotein synthesis.
- The downregulation of selenoproteins was accompanied by general inhibition of protein synthesis.

Abbreviations: glutathione peroxidase 2 (GPX2); thioredoxin reductase-1 (TXNRD1); antioxidant response element (ARE)

Correspondence:

Paul R. Copeland

Department of Biochemistry and Molecular Biology

Rutgers - Robert Wood Johnson Medical School

675 Hoes Ln, Piscataway, NJ 08854 USA

E-mail address: paul.copeland@rutgers.edu

Disclosures: The authors do not have any financial professional or personal conflicts to disclose

Preprint server: https://doi.org/10.1101/2023.05.12.540527

Abstract

Background and Aims: Many of the proteins that contain the amino acid selenocysteine are required for optimal defense against cellular stress. As such, one might expect selenoprotein synthesis to persist or be induced upon cellular insult. Because selenocysteine is incorporated by a complex post-transcriptional mechanism, monitoring the transcription of selenoprotein genes is not adequate to understand the regulation of selenoprotein synthesis. We aimed to determine whether selenoprotein synthesis is regulated by the induction of hepatotoxic stress.

Methods: We used hepatotropic clinically relevant drugs to evaluate the regulation of selenoprotein synthesis in human hepatocarcinoma cells.

Results: We found that two drugs, benzbromarone and sorafenib, caused significant inhibition of selenoprotein synthesis. However, the loss of selenoprotein expression was not specific as total protein synthesis was similarly down-regulated only by benzbromarone and sorafenib.

Conclusions: These results allow us to conclude that these hepatotoxins do not induce or preserve selenoprotein synthesis as a protective mechanism.

Keywords:

Benzbromarone; hepatotoxin; selenocysteine; selenoproteins

1. Introduction

The trace element selenium is an essential component of 25 human proteins as the amino acid selenocysteine (Sec). Many of these selenoproteins are oxido-reductases that remediate cellular damage due to oxidation. Additionally, these proteins are involved in a myriad of other functions including thyroid hormone production, protein folding and lipid metabolism[1]. Some selenoprotein genes are transcriptionally upregulated in response to stress. For example glutathione peroxidase 2 (GPX2) and thioredoxin reductase-1 (TXNRD1) transcription is upregulated by ~10 and ~2-fold, respectively, as a result of concanavalin A induced liver injury in mice[2]. This effect is mediated through the antioxidant response pathway via NRF2 stabilization and binding to promoters with the antioxidant response element (ARE)[3–5]. However, since selenoprotein synthesis is also regulated at the translational level due to the complex process of Sec incorporation[6], we sought to determine how the translation of selenoprotein mRNAs is affected by drug treatment in a hepatoma cell line model (HepG2). In this study, we used metabolic labeling with radioactive selenium to determine whether a selection of drugs with known hepatotoxicity[7] or the liver tropism regulate selenoprotein synthesis. We also measured cell viability and NRF2 activation with an ARE reporter. Surprisingly, we found no evidence of induced selenoprotein expression by this group of drugs, but two of them, benzbromarone and sorafenib, significantly downregulated both selenoprotein and total protein synthesis.

2. Materials and Methods

2.1 Cell culture and reagents

All of the experiments in which endogenous and exogenous selenoprotein expression was assessed were performed in HepG2 cells grown in EMEM (Life Technologies) plus 10% fetal bovine serum (except where indicated). HepG2 cells stably expressing the pARE-T1-Luciferase (ARE-LUC) construct[10,11] were obtained from A.N. Kong, Rutgers School of Pharmacy. Hepatotoxins and controls used in this study were all purchased from Millipore Sigma: Benzbromarone, Clozapine, Allopurinol, Sorafenib, D-Mannitol, and Ritonavir. Each of them was reconstituted to either 1 mM or 10 mM stock in DMSO.

2.2 Metabolic radiolabeling

WT or ARE-LUC expressing HepG2 were grown in either 24-well or 96-well plates to approximately 70% confluence when the medium was changed to serum-free medium supplemented with 100 nM ^{75}Se (University of Missouri Research Reactor; specific activity of ~500 Ci/g) or 50 uCi/ml 35S Met/Cys (Perkin Elmer). For drug or control treatments, the stocks were diluted to the indicated concentrations with serum free media containing 100 nM ^{75}Se before being added to cells. After 24 hours of labeling, cells were lysed in NP40 cell lysis buffer (10mM Tris-Cl, pH 7.4, 1% NP40, 1X Roche Complete protease inhibitors) at 4C for 1 hour followed by centrifugation at 18,000 g for 10 mins and supernatant collected as sample for analysis. Samples were diluted with 4X SDS sample buffer (100 mM Tris-HCl pH 6.8, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue and 20% (v/v) glycerol) and boiled at 95 °C for 5 minutes and spun at max speed for 1 minute. Proteins were resolved by 4-15% gradient SDS gels (Biorad). The gels were dried and exposed to a phosphorimage screen for 24 hrs before being developed on the Typhoon FLA7000 (Cytiva).

2.3 Cell viability assay

Cell viability was assessed using alamarBlue reagent (Thermo Fisher Scientific). HepG2 cells in 96 well plates were treated with compounds indicated in Figure 2 in 90 µl of serum-free media supplemented with ^{75}Se for 6 hours when 10 ul of alamarBlue reagent was added and fluorescence readings were taken (excitation/emission-560/590). A plot of relative fluorescence units vs. drug concentration was used to determine if cell viability was altered.

4.4 ARE activation assay

Cells expressing the ARE-LUC construct were treated with the compounds indicated in Figure 1 for 24 hours and then lysed in NP40 lysis buffer and centrifuged at 18,000 g for 10 minutes. 50ul of the supernatant was used to analyze changes in luminescence readings compared to untreated control cells using a plate luminometer (Berthold).

3. Results and Discussion

3.1 ^{75}Se Metabolic labeling reveals downregulation of selenoprotein synthesis.

In order to determine the effect of clinically relevant drugs with hepatotoxic potential on selenoprotein

synthesis, we metabolically labeled HepG2 cells with ^{75}Se-selenite simultaneously with drug treatment ranging from 2.5 to 50 μM for 24 hours. We used three compounds with known hepatotoxicity: 1) the xanthine oxidase inhibitor benzbromarone, 2) the protease inhibitor ritonavir, 3) the antipsychotic clozapine, and one drug with known liver tropism, the chemotherapeutic sorafenib. We also included two controls, D-mannitol and allopurinol, the latter of which is another xanthine oxidase inhibitor that is less toxic[12]. These drugs were chosen for their range of responses in liver, including known activation of the NRF2 response pathway. In addition, liver is a tissue that displays robust selenoprotein production, so regulation at the translational level should be readily apparent. Selenoprotein synthesis was evaluated by SDS-PAGE of cell lysates followed by Phosphorimaging (Figure 1A). While we saw no evidence of enhanced selenoprotein synthesis, benzbromarone and sorafenib were both strongly inhibitory at 5 and 10 μM, respectively. This inhibition was also apparent after 3 hours of treatment (Figure 1B). It is notable that Benzbromarone seems to differentially inhibit the lower molecular weight (MW) selenoproteins (including the GPXs) relative to the predominant upper molecular weight protein, which is thioredoxin reductase (TXNRD1)[13]. This effect was not observed with Sorafenib treatment, which seems to inhibit selenoprotein synthesis uniformly (Figure 1A, compare 5 μM Benzbromarone to 10 μM Sorafenib). In a similar fashion, the preservation of TXNRD1 synthesis is known to occur under conditions of selenium deficiency[14], and it is thought to be driven by changes in the amount of methylated Sec tRNA[15]. This finding may suggest that the two drugs inhibit selenoprotein synthesis via two different pathways but further quantitative study is necessary.

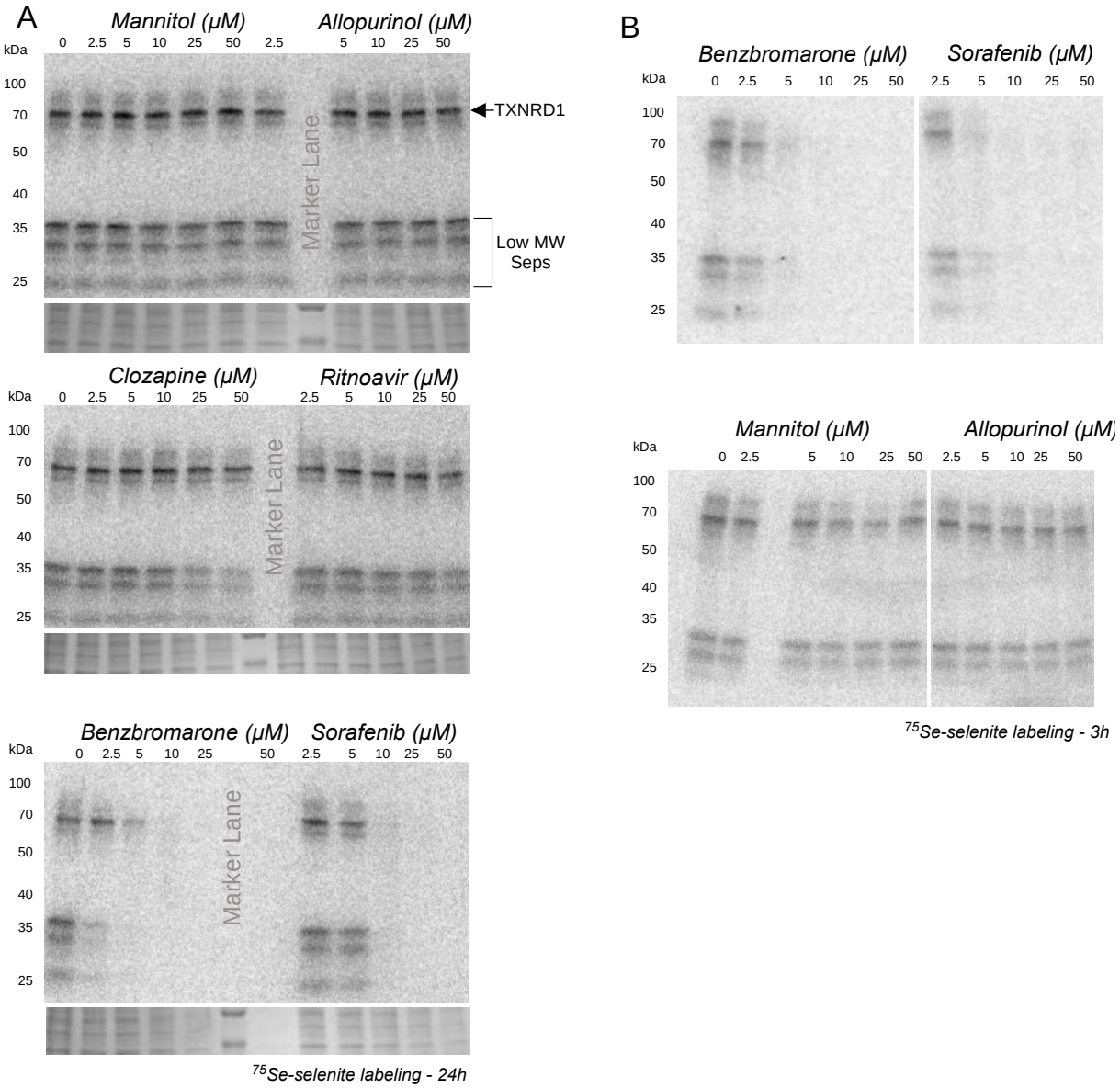
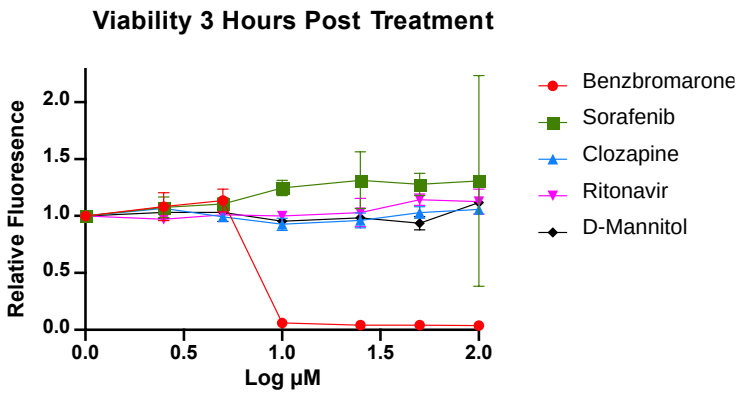


Fig. 1. Benzbromarone and sorafenib are potent inhibitors of selenoprotein synthesis in HepG2 cells. A). HepG2 cells were grown for 24 hours in the presence of 100 nM ^{75}Se-selenite and the indicated concentrations of drugs. Cells were lysed and cytoplasmic proteins resolved by SDS-PAGE. Gels were stained with Coomassie Blue (lower panels) and radioactive selenoproteins were detected by phosphorimaging (upper panels). B) Same as in A) except metabolic labeling was for 3 hours.

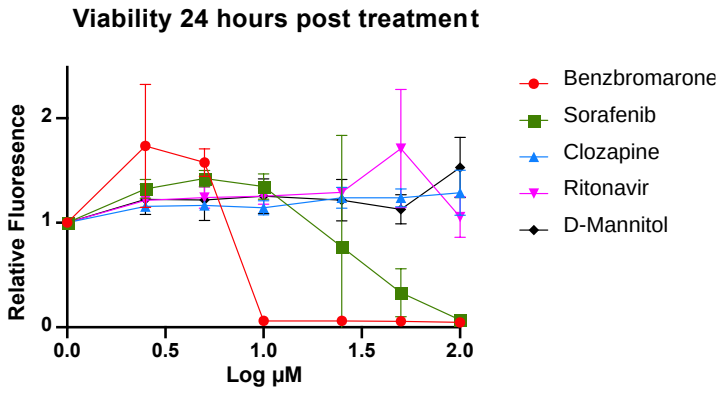
3.2 Cell death does not drive reduction of selenoprotein synthesis.

Although the cells appeared to be healthy in the low inhibitory concentrations of benzbromarone and sorafenib, we sought to determine the effect on viability with the alamarBlue cytotoxicity assay. HepG2 cells were grown to ~70% confluence and switched to serum-free medium containing drug concentrations ranging from 2.5 to 25 uM. Viability was assessed at 24 hours post-treatment by alamarBlue staining. Figure 2A shows that none of the cells were sensitive to these drugs at the 3-hour time point but that complete cell death was observed at the 10 μM Benzbromarone concentration after 24 hours of treatment (Figure 2B). We therefore observed no indication that cell death is the primary driver behind the inhibition of selenoprotein synthesis.

A



B



C

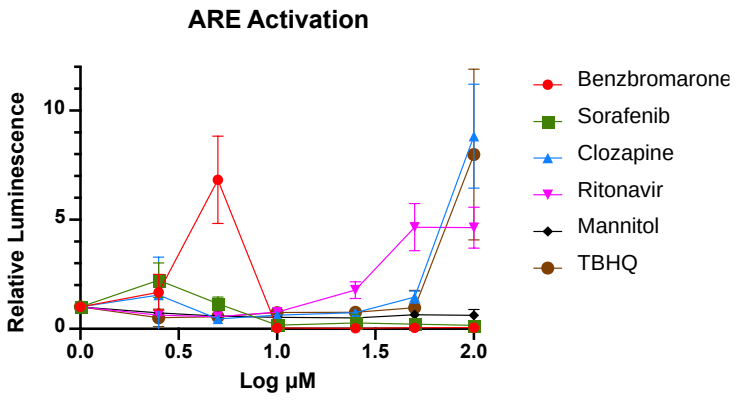


Fig. 2. Benzbromarone shows cytotoxicity and ARE activation. A) HepG2 cells were grown in the indicated drug concentrations for 3 hours followed by alamarBlue staining. Fluorescence was plotted versus drug concentration and the results from 3 biological replicates are shown. B) Same as in A except drug treatment was for 24 hours. C) HepG2 cells stably transfected with the ARE-LUC construct were treated with the indicated drug concentrations. TBHQ was included as the postive control for ARE activation. Luminescence was plotted versus drug concentration and the results from 3 biological replicates are shown as the average +/- standard deviation.

3.3 NRF2 is activated by benzbromarone

In addition to viability, we also wished to determine the extent to which each of these drugs could activate the antioxidant response element (ARE) pathway driven by NFE2L2 (NRF2). For this experiment we obtained HepG2 cells stably expressing a luciferase construct driven by an ARE-containing promoter[16]. These cells were treated with drug concentrations ranging from 2.5 to 100 μM, which revealed ARE activation at 5 μM for benzbromarone, and 100 μM for the positive control, TBHQ (Figure 2C). No ARE activation was observed for sorafenib or the negative control, D-mannitol. Together these results establish that the loss of selenoprotein synthesis may correlate with NRF2 pathway activation for benzbromarone but not for sorafenib. This is consistent with the selective preservation of TXNRD1 expression during benzbromarone but not sorafenib treatment as shown in Figure 1A.

3.4 ^{35}S Met labeling reveals a general effect on protein synthesis

To determine whether the inhibition of selenoprotein synthesis is specific, we performed similar experiments using ^{35}S Met/Cys labeling to observe total new protein synthesis. For this experiment we trace-labeled the cells with a mixture of ^{35}S Met and Cys in the presence of the lower range of benzbromarone, sorafenib and D-mannitol. Figure 3A shows that total protein synthesis appears to be inhibited in a similar fashion as selenoprotein synthesis. Quantitative analysis (Figure 3C) confirms that ^{35}S incorporation is inhibited to the same extent as ^{75}Se for both benzbromarone and sorafenib. Consistent with the prior data, benzbromarone is a more potent inhibitor with substantial loss of signal at 2.5 and 5 μM whereas sorafenib inhibition is only apparent at 10 μM. These results indicate that the inhibition of selenoprotein synthesis is not specific and is likely the result of activation of a stress pathway that inhibits total protein synthesis.

Figure 3 Shetty et al

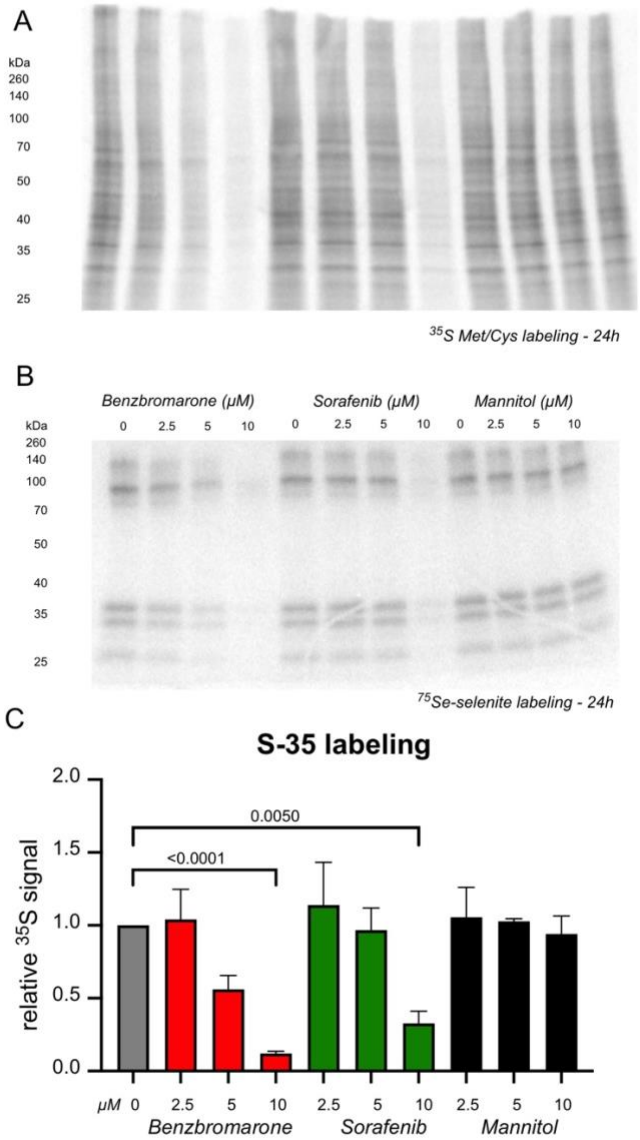


Fig. 3. Benzbromarone and sorafenib inhibit total protein synthesis. A) HepG2 cells were grown for 24 hours with the indicated drug concentrations and in the presence of 50 uCi/ml ^{35}S Met/Cys mix plus 100 nM cold sodium selenite. Cells were lysed and cytoplasmic proteins resolved by SDS-PAGE. Gels were dried and radioactive proteins were detected by phosphorimaging. B) In parallel with the experiment descrbied in A), cells were labeled with 100 nM ^{75}Se-selenite. C) Quantification of total radioactivity in each lane of the 35S labeling gel shown in A). The values were derived from three biological replicates.

4. Conclusions

Here we have demonstrated that the cellular response to the liver tropic drugs tested does not include translational induction of selenoprotein synthesis. Through the use of metabolic labeling with ^{75}Se-selenite, we directly evaluated the extent of Sec incorporation after drug treatment, providing a sensitive and specific evaluation of selenoprotein synthesis. By monitoring selenoprotein synthesis with metabolic labeling, we have found that there is no indication of enhanced selenoprotein production under stress conditions induced by hepatotoxic drugs. This finding stands in contrast to the fact that selenoprotein mRNA levels significantly increase upon activation of the NRF2 pathway[2].This raises the possibility that there may be a disconnect between mRNA and selenoprotein induction as a result of NRF2 activation, which will need further exploration. These results also have implications about the potential side effects of clinically relevant drugs, especially benzbromarone, which is still in use in some parts of the world.

Declarations

No ethical approval was required as this study did not involve human participants or laboratory animals

Data availability statement

Data associated with this study been deposited into a publicly available repository: BioRxv doi: https://doi.org/10.1101/2023.05.12.540527

Acknowledgements

Thanks go to Dr. Lauren Aleksunes (Rutgers School of Pharmacy) for initial discussions about hepatotoxins and suggestions. Thanks also to Dr. A.N. Kong (Rutgers School of Pharmacy) for providing the ARE-LUC construct.

Funding: This research was supported by grants from the National Institute for General Medical Sciences and National Institute of Environmental Health Sciences, R01GM077073 (PRC) and R21ES032863 (SPS and PRC), respectively.

References;

1. Labunskyy VM, Hatfield DL, Gladyshev VN. Selenoproteins: molecular pathways and physiological roles. Physiol Rev. 2014 Jul;94(3):739–777. PMCID: PMC4101630

2. Osburn WO, Yates MS, Dolan PD, Chen S, Liby KT, Sporn MB, Taguchi K, Yamamoto M, Kensler TW. Genetic or pharmacologic amplification of nrf2 signaling inhibits acute inflammatory liver injury in mice. Toxicol Sci. 2008 Jul;104(1):218–227. PMCID: PMC2435415

3. Herpers B, Wink S, Fredriksson L, Di Z, Hendriks G, Vrieling H, de Bont H, van de Water B. Activation of the Nrf2 response by intrinsic hepatotoxic drugs correlates with suppression of NF-κB activation and sensitizes toward TNFα-induced cytotoxicity. Arch Toxicol. 2016 May;90(5):1163–1179. PMCID: PMC4830895

4. Björnsson ES. Hepatotoxicity by Drugs: The Most Common Implicated Agents. Int J Mol Sci. 2016 Feb 6;17(2):224. PMCID: PMC4783956

5. Baird L, Yamamoto M. The Molecular Mechanisms Regulating the KEAP1-NRF2 Pathway. Mol Cell Biol [Internet]. 2020 Jun 15;40(13). Available from: http://dx.doi.org/10.1128/MCB.00099-20 PMCID: PMC7296212

6. Gonzalez-Flores JN, Shetty SP, Dubey A, Copeland PR. The molecular biology of selenocysteine. Biomol Concepts. 2013 Aug;4(4):349–365. PMCID: PMC4269102

7. Suzuki A, Andrade RJ, Bjornsson E, Lucena MI, Lee WM, Yuen NA, Hunt CM, Freston JW. Drugs associated with hepatotoxicity and their reporting frequency of liver adverse events in VigiBase: unified list based on international collaborative work. Drug Saf. 2010 Jun 1;33(6):503–522. PMID: 20486732

8. Sivera F, Andres M, Dalbeth N. A glance into the future of gout. Ther Adv Musculoskelet Dis. 2022 Jul 28;14:1759720X221114098. PMCID: PMC9340313

9. Roos NJ, Duthaler U, Bouitbir J, Krähenbühl S. The uricosuric benzbromarone disturbs the mitochondrial redox homeostasis and activates the NRF2 signaling pathway in HepG2 cells. Free Radic Biol Med. 2020 May 20;152:216–226. PMID: 32198009

10. Yu R, Mandlekar S, Lei W, Fahl WE, Tan TH, Kong ANT. p38 Mitogen-activated Protein Kinase Negatively Regulates the Induction of Phase II Drug-metabolizing Enzymes That Detoxify

Carcinogens*. J Biol Chem. 2000 Jan 28;275(4):2322–2327.

11. Wasserman WW, Fahl WE. Functional antioxidant responsive elements. Proc Natl Acad Sci U S A. 1997 May 13;94(10):5361–5366. PMCID: PMC24683

12. Reinders MK, Haagsma C, Jansen TLTA, van Roon EN, Delsing J, van de Laar MAFJ, Brouwers JRBJ. A randomised controlled trial on the efficacy and tolerability with dose escalation of allopurinol 300-600 mg/day versus benzbromarone 100-200 mg/day in patients with gout. Ann Rheum Dis. 2009 Jun;68(6):892–897. PMID: 18633127

13. Shetty S, Marsicano JR, Copeland PR. Uptake and Utilization of Selenium from Selenoprotein P. Biol Trace Elem Res. 2018 Jan;181(1):54–61. PMCID: PMC5680150

14. Howard MT, Carlson BA, Anderson CB, Hatfield DL. Translational redefinition of UGA codons is regulated by selenium availability. J Biol Chem. 2013 Jul 5;288(27):19401–19413. PMCID: PMC3707644

15. Carlson BA, Xu XM, Gladyshev VN, Hatfield DL. Selective rescue of selenoprotein expression in mice lacking a highly specialized methyl group in selenocysteine tRNA. J Biol Chem. 2005 Feb 18;280(7):5542–5548. PMID: 15611090

16. Saw CLL, Yang AY, Guo Y, Kong ANT. Astaxanthin and omega-3 fatty acids individually and in combination protect against oxidative stress via the Nrf2-ARE pathway. Food Chem Toxicol. 2013 Dec;62:869–875. PMID: 24157545