Cefadroxil potency as cancer co-therapy candidate by glutathione s-transferase mechanism

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Background: Glutathione S-transferases (GSTs) have an important role in the detoxification of electrophiles, such as some anticancer drugs. Compounds with phenolic and/or α,β-unsaturated carbonyl group have been known as GSTs inhibitor *in vitro*. Cefadroxil *in vitro* decreased GST-Pi activity but not GSTs in rat kidney cytosol. GST inhibitor in a specific organ and of a specific class is needed for safety in cancer chemotherapy. The study aims to observe the effect of cefadroxil on GSTs *in vivo* in rat kidney cytosol and then compare it to those seen for liver, lung, and spleen *in vivo*.

Methods: Cefadroxil was given twice a day by forcefeeding for five days. Rat kidney cytosol was then prepared and its protein concentration was determined. Cytosolic total GST, GST-Mu and GST-Pi activities were monitored by a continuous spectrophotometric method using the following substrates: 1-chloro, 2,4-dinitrobenzene (CDNB) (non-specific substrate), 1,2-dichloro-4-nitrobenzene (DCNB) for GST-Mu, and ethacrynic acid (EA) for GST-Pi.

Results: The data showed that cefadroxil significantly increased the activity of GSTs, GST-Mu, and GST-Pi in rat kidney cytosol (8.75%, 47.81%, and 6.67% respectively).

Conclusion: Cefadroxil did not inhibit GSTs, GST-Mu, and GST-Pi in rat kidney in vivo indicating that it does not inhibit chemotherapy detoxification by GSTs, GST-Mu, and GST-Pi in normal kidney cells.

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Introduction

In the last two centuries, living organisms tend to be exposed to carcinogens because of pollution.[1] The number of deaths caused by cancers continue to increase, and they are the leading cause of death in some western countries.[2] Cells try to protect themselves from xenobiotic pollutants, either electrophiles or reactive oxygen species, by inducing detoxifying phase II enzyme.[3,4]

GSTs play a major rule among phase II detoxifying enzymes, catalysing conjugation reaction between electrophiles and GSH resulting in a more hydrophilic compound.[3] Cytosol is the major source of this enzyme, besides mitochondria, microsome, and nucleus.[3,5] Because of its significant role, many studies have been conducted on this enzyme in drug discovery, such as studies on anti-helminthic and anti-cancer drug candidates.[6,7]

In contrast to the normal physiology whereby GSTs induction protects the body against toxic compounds, induction of GSTs worsened chemotherapy. Chemotherapy resistance occurs because of GST-Pi induction during cancer development. GSTs blocks c-jun-N-terminal kinase (JNK) directly and catabolize H_2O_2, resulting in apoptosis failure.[7] In addition, some anti-cancer compounds act as substrates for GSTs, resulting in products which are less active and more hydrophile.[3,8] In the contrary, a prodrug which is activated by specific GSTs will give a specific action to specific cancer cell by specific enzyme induction.[9]

Many inducers effect GST genes through the antioxidant-responsive element (ARE), the xenobiotic-responsive element (XRE), the GST P enhancer 1(GPE), or the glucocorticoid-responsive element (GRE). The mechanisms include transcriptional activation, stabilisation of either mRNA or protein, and gene amplification.[10]

GST induction due to increasing its expression through electrophilic centre influence, either as C, N, or S has been studied.[5,11] N^+ cation inhibits Keap1, a protein which plays an important role in inhibiting Nrf2 binding to GPE. When Keap1 is inhibited by N^+ cation, Nrf2 can bind to GPE resulting in transcription. Nrf2 acts as transcription factor which increase GSTs expression during the five days of PVP treatment.

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Without GSTs inducer, Keap1 will bind to Nrf2 in cytoplasm and inhibit Nrf2 movement to nucleus. Jun, Fos, and large Maf are involved in GST-Pi gene transcription.[12]

Meanwhile, inhibition of GSTs may occur either when cefadroxil binds to the H site of GSTs through hydrophobic bonds or N^+ cations from cefadroxil mesomery binds GS^- anion resulting in lower GSH conjugates.[13]

In addition, more GSH conjugates (products) are results of either lower product affinity by covalent enzyme modification or inhibition of enzyme degradation pathways.[14] In this case, the occurrence of more GSH conjugates may suggest the presence of more GS^- anions due to oxygen anions of cefadroxil and PVP abstracting hydrogen of GSH.[13]

GST inhibitor in a specific organ and of a specific class is needed for safety in cancer chemotherapy. To our knowledge, there are limited compounds which are active as GST inhibitors.[5] Some compounds containing phenolic and α, β-unsaturated carbonyl groups inhibit GSTs activity *in vitro*. Cefadroxil has these properties and inhibits GST-Pi in rat kidney *in vitro*.[15] We predict that it could inhibit GSTs *in vivo*. This study was conducted to determine whether cefradroxil is a good GSTs inhibitor *in vivo*, in our efforts to find a co-therapeutic agent for cancers with increased GSTs activity.

Cefadroxil, a parahydroxy cephalexine derivative, is almost unmetabolised because 90% of cephalexin were found to be excreted unchanged in urine.[16] Meanwhile, PVP (approx. 11,500 – 25,000 MW) was found to be unchanged in urine (unmetabolised) and accumulated in rat kidney mitochondria.[17]

Materials and Methods

Animals

Wistar rats (3 months-old) were obtained from Sanggar Kegiatan Belajar (SKB) Yogyakarta, Indonesia. They were maintained in acontrolled environment, given water *ad libitum* and laboratory pellets from Pharmacology Laboratory, Faculty of Pharmacy, UGM, Indonesia.

Materials

Cefadroxil was purchased from ACS Dobfar, Tribiano Milano, Italy. L-glutathione (GSH), bovine serum albumin (BSA), and ethacrynic acid (EA) was purchased from Sigma Chem. Co, Western Australia. 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB) was purchased from Aldrich, St. Louis, US. PVP, ethanol, methanol, KH_2PO_4, K_2HPO_4 were purchased from E. Merck, Darmstadt, Germany. Protein determination (Kit) was purchased from Bio-Rad Laboratories, Hercules, CA, US.

Animals and treatments

Male Wistar rats (3 months old) weighing 200 ± 20 g were maintained at room temperature and a normal photoperiod of 12 h of darkness and 12 h of light. Animals were randomly divided into 3 groups of 10 (ten) each and maintained in our animal facility for 1 week. Rats were force-fed for 5 days, given aquadest (control), PVP 0.5% b/v solution (solvent control), and cefadroxil 90 mg/kg bw in PVP solution (treatment).

Tissue preparation

At the end of the experimental period, rats were starved overnight and then sacrificed by cervical dislocation. Tissues were removed, rinsed, placed, and homogenised in cold 0.1 M potassium phosphate buffer (pH 7.5), for 5 min. Each sample was centrifuged at 10,000 x g for 30 min in 4°C; the pellet was discarded and the supernatant centrifuged at 105,000 x g for 90 min at 4°C. The resulting supernatant (cytosol) was stored at -20°C until used. This was done based on the method of Reddy et al with modification.[18]

Protein assay

Protein concentration was approximated using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA), based on the Bradford method.[19] Bovine

serum albumin was used as the protein standard, and spectrophotometric measurements were made at 595 nm using a Genesys 5 Milton Roy spectrophotometer.

Enzyme assays

These were done based on the method described by Habig and Jakoby et al with modification.[20]

Cytosolic total GST activity

The reaction was initiated by the addition 0.1 M potassium phosphate buffer (pH 6.5) 700.0 µl, cytosol 20.0 ml, 50 mM GSH 15.0 ml (in aquadest), and 50 mM CDNB 15.0 ml (in ethanol) into 1 ml cuvette. After mixing, the formation of GSH conjugate was monitored at 340 nm at 4°C; the reaction had to be linear for at least three min. to be included in the calculation of the specific activity. The measurements (Abs/min) were made using a Genesys 5 Milton Roy spectrophotometer.

GST-Mu activity

The reaction was initiated by the addition 0.1 M potassium phosphate buffer (pH 7.5) 620.0 µl, cytosol 40.0 µl, 50 mM GSH 75.0 µl (in aquadest), and 50 mM DCNB 15.0 µl (in ethanol) into 1 ml cuvette. After mixing, the formation of GSH conjugate was monitored at 345 nm at 4°C; the reaction had to be linear for at least three min. to be included in the calculation of the specific activity. The measurements (Abs/min) were made using a Genesys 5 Milton Roy spectrophotometer.

GST-Pi activity

The reaction was initiated by the addition 0.1 M potassium phosphate buffer (pH 6.5) 695.25 µl, cytosol 20.0 µl, 10 mM GSH 18.75 µl (in aquadest), and 10 mM EA 15.0 µl (in ethanol) into 1 ml cuvette. After mixing, the formation of GSH conjugate was monitored at 270 nm at 4°C; the reaction had to be linear for at least three min. to be included in the calculation of the specific activity. The measurements (Abs/min) were made using a Genesys 5 Milton Roy spectrophotometer.

Enzyme analysis

GSH conjugation rate (V) was calculated as follows:

$$V = \frac{(\text{abs/min}) / \varepsilon . \text{ cuvette thickness}}{\text{protein concentration}}$$

$\varepsilon_{GS\text{-}DNB}$ pada λ 340 = 9.6 mM^{-1}
$\varepsilon_{GS\text{-}CNB}$ pada λ 345 = 8.5 $mM^{-1}cm^{-1}$
$\varepsilon_{GS\text{-}AE}$ pada λ 270 = 5 $mM^{-1}cm^{-1}$

$$\% \text{ inhibition} = \frac{\text{Vcontrol} - \text{V treatment}}{\text{Vcontrol}}$$

Statistical analysis

A normality test was performed for testing if the data was sampled from populations that follow Gaussian distributions. This assumption was tested using the Kolmogorov and Smirnov method. Statistical analysis of data was performed by means of parametric (one-way) ANOVA. Where significant overall differences ($p < 0.05$) were observed, further analysis among experimental groups was performed using Tukey multiple-range test.

Results

As seen from Figure 1, the protein concentrations were found to decrease for both cefadroxil in PVP solution (18.75%) and PVP solution alone (12.5%).

Meanwhile, in calculating percentage of enzyme induction or inhibition (Table 1), correction by PVP was done because PVP itself increased GSTs activity compared to control ($p<0.05$).

The data showed that cefadroxil significantly increased the activity of GSTs, GST-Mu, and GST-Pi in rat kidney cytosol (8.75%, 47.81%, and 6.67% respectively), as well as PVP (12.86%, 25.90%, and 3.63% respectively) (Figures 2, 3 and 4).

Discussion

The significant induction of GST-Mu and GST-Pi by cefadroxil *in vivo* observed in this study was not in agreement with previous reports.[15,21] We suggest that

the induction occurred because of increased enzyme quantity during five days of treatment and that the induction mechanism is due to mesomery (Fig. 5). Cefadroxil has two potential electrophilic centres resulting in stronger induction than PVP which has only one. But, the validation of this suggestion requires crystal structure observation.[6]

Cefadroxil induction on GSTs, GST-Mu, and GST-Pi *in vivo* were in contrast with a previous *in vitro* report.[15] This is probably due to its metabolism. Similar report also was found in curcumin that it inhibited GSTs *in vitro*[22] but not *in vivo*[2]. Both cefadroxil and curcumin have similar molecular structures, which are phenolic compounds and have α,β- unsaturated carbonyl groups.

Table 1 shows the *in vivo* effects of cefadroxil on the other organs. Cefadroxil has been found to induce GSTs (90.85%), GST-Mu (38.33%), and GST-Pi (34.03%) in spleen.[23] In lung, it also induced GSTs (43.57%) and GST-Mu (8.21%), but inhibited GST-Pi (11.7%).[24] Meanwhile, it inhibited GST-Mu (27.5%) and GST-Pi (6.08%), but induced GSTs (17.58%) in liver.[13]

Differences in physicochemical properties of each organ influence cefadroxil effects. It is accumulated more in liver and excreted faster in kidney.

In conclusion, cefadroxil has potency to be developed as co-therapy compound on chemotherapy because it can inhibit GSTs of a specific class and in a specific organ so that it gives lower toxicity. However, further study on its inhibition mechanism *in vivo* is needed.

Conclusion

Cefadroxil did not inhibit GSTs, GST-Mu, and GST-Pi in rat kidney *in vivo* indicating that it does not inhibit chemotherapy detoxification by GSTs, GST-Mu, and GST-Pi in normal kidney cell.

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REFERENCES

1. Sheehan D, Meade G, Foley VM, Dowd CA. Structure, Function and Evolution of Glutathione Transferases: Implications for Classification of Non-mammalian Members of An Ancient Enzyme Superfamily. Biochem J 2001; 360: 1–16.
2. Sharma RA, Mclelland HR, Hill KA, Ireson CR, Euden SA, Manson MM, Pirmohamed M, Marnett LJ, Gescher AJ, Steward WP. Pharmacodynamic and Pharmacokinetic Study of Oral Curcuma Extract in Patient with Colorectal Cancer. Clin Cancer Res 2001; 7: 1894-900.
3. Aliya S, Reddanna P, Thyagaraju K. Does Glutathione S-transferase Pi (GST-Pi) A Marker Protein for Cancer? Mol Cell Biochem 2003; 253: 319-27.
4. Dinkova-Kostova AT, Massiah MA, Bozak RE, Hicks RJ, Talalay, P. Potency of Michael Reaction Acceptor as Inducers of Enzymes that Protect against Carcinogenesis Depends on Their Reactivity with Sulfhydryl Groups. Proc Nat Acad Sci 2000; 98 (6): 3404-9.
5. Eaton DL, Bammler TK. Concise Review of the Glutathione S-transferase and Their Significance to Toxicology. Toxicol Sci 1999; 49: 156-64.
6. Jao S, Chen J, Yanga K, Li W. Design of Potent Inhibitors for Schistosoma japonica Glutathione S-transferase. Bioorganic Med Chem 2006; 14: 304–18.
7. Zhao G, Yu T, Wang R, Wang X, Jing Y. Synthesis and Structure–activity Relationship of Ethacrynic Acid Analogues on Glutathione-s-transferase P1-1 Activity Inhibition. Bioorganic Med Chem 2005; 13: 4056–62.
8. Stavrovskaya AA. Cellular Mechanisms of Multidrug Resistance of Tumor Cells. Biochem. (Moscow) 2000; 65 (1): 95-106.
9. Chakrapani H, Kalathur RC, Maciag AE, Citro ML, Ji X, Keefer LK, Saavedra, JE. Synthesis, Mechanistic Studies, and Anti-proliferative Activity of Glutathione/glutathione S-transferase-activated Nitric Oxide Prodrugs. Bioorg Medicinal Chem 2008; 16: 9764–9771.
10. Hayes JD, Pulford DJ. The Glutathione S-Transferase Supergene Family : Regulation of GST and the Contribution of Isoenzyme to Cancer Chemoprotection and Drug Resistance. Crit. Rev. in Biochem. and Mol Biol.1995; 30 (6): 445-600.
11. Ikeda H, Nishi S, Sakai M. Transcription Factor Nrf2/Mafk Regulates Rat Placental Glutathione S-transferase Gene During Hepatocarcinogenesis. Biochem J 2004; 380(Pt 2): 515-521.
12. Ikeda H, Serria SM, Kakizaki I, Hatayama I, Satoh K, Tsuchida S, Muramatsu M, Nishi S, Sakai M. Activation of Mouse Pi-class Glutathione S-transferase Gene by Nrf2 (NF-E2-related factor 2) and Androgen. Biochem J 2002; 364: 563-570.
13. Putroutomo MY. Effect of Cefadroxil on Rat Liver GSTs, GST-Mu and GST-Pi Activity in (*In Vivo*), Thesis 2007; Faculty of Pharmacy, Yogyakarta: UGM (*in Indonesian*).
14. Matthews H.R. 1996. Cell and Molecular Biology: Enzyme Activation and Inhibition, Lecture #6, web.macam98.ac.il, accessed: 15 December 2006.

15. Octaviani E. Effect of Cefadroxil on Rat Kidney GSTs and GST-Pi Activity in (*In Vitro*), Thesis 2006; Faculty of Pharmacy, Yogyakarta: UGM (*in Indonesian*).
16. Drug Bank. 2006. Cephalexin. http://www.drugbank.cgi, accessed 16 December 2006.
17. FAO, WHO. 1967. Toxicological Evaluation of Some Antimicrobials, Antioxidants, Emulsifiers, Stabilizers, Flour Treatment Agents, Acids, and Bases. http://www.inchem.org, accessed 10 February 2007.
18. Reddy KV, Anuradha D, Kumar TC, Reddanna P, Induction of Ya_1 Subunit of Rat Hepatic Glutathione S-transferase by Exercise-induced Oxidative Stress, Arch Biochem and Biophys 1995; 323: 6-10.
19. Bradford MM, A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein Dye Binding, Anal Biochem 1976; 72: 248-54.
20. Habig WH, Jakoby WB. Assays for Differentiation of Glutathione S-transferase. Meth Enzymol 1981; 77: 398-405.
21. Dewi N. Effect of Cefadroxil on Rat Liver GST-Mu and GST-Pi Activity in (*In Vitro*), Thesis 2006; Faculty of Pharmacy, Yogyakarta: UGM (*in Indonesian*).
22. Martono S. Effect of Methoxyphenyl Derivatives on Rat Liver GSTs Activity. Research Report 2000; Research Institute of Yogyakarta: UGM (*in Indonesian*).
23. Tjipto SS. Effect of Cefadroxil on Rat Spleen GSTs, GST-Mu and GST-Pi Activity in (*In Vivo*), Thesis 2007; Faculty of Pharmacy, Yogyakarta: UGM (*in Indonesian*).
24. Indartono IDR. Effect of Cefadroxil on Rat Lung GSTs, GST-Mu and GST-Pi Activity in (*In Vivo*), Thesis 2007; Faculty of Pharmacy, Yogyakarta: UGM (*in Indonesian*).

Table 1: Effects of Cefadroxil *in vivo* on GST activity in kidney, liver, lung, and spleen.

Organ	General Class (%)	Mu Class (%)	Pi Class (%)
Kidney	+ 8.75	+ 47.81	+ 6.67
Liver[13]	+ 17.58	- 27.50	- 6.08
Lung[24]	+ 43.57	+ 8.21	- 11.17
Spleen[23]	+ 90.85	+ 38.33	+ 34.03

Notes: (+) value showed GST induction, (-) value showed GST inhibition

Figure 1: Protein concentration of rat kidney cytosol

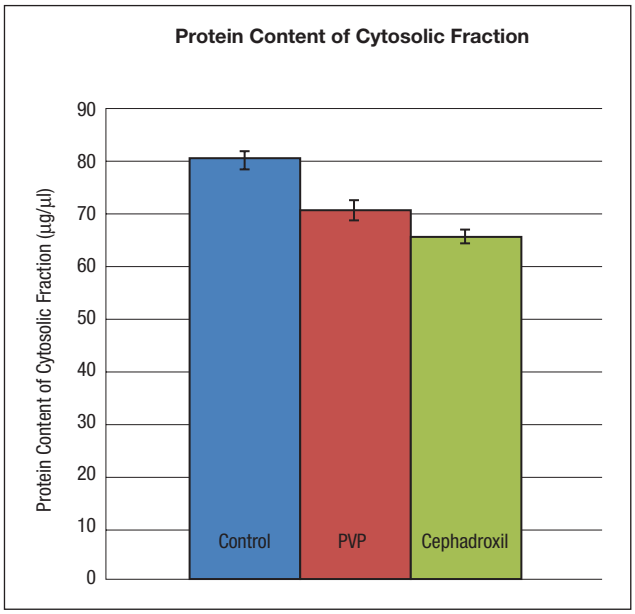

Figure 2: GSTs activity of rat kidney cytosol

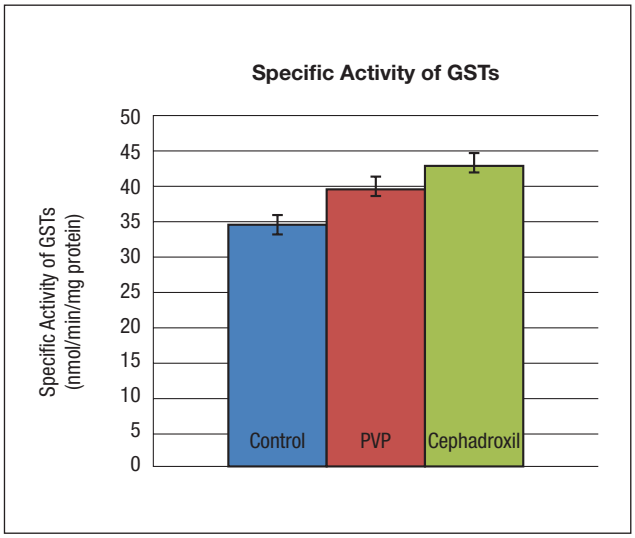

Figure 3: GST-Mu activity of rat kidney cytosol

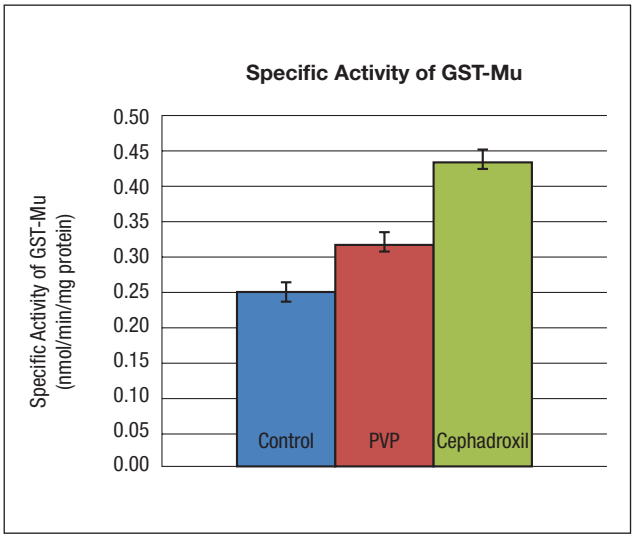

Figure 4: GST-Pi activity of rat kidney cytosol