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[^{35}S]*S*-[5-(4-benzoylphenyl)pentyl]glutathione: A Versatile Radioligand Targeting *GS-X* Pumps with the Ability of Photoaffinity Labeling

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Abstract

[^{35}S]*S*-[5-(4-benzoylphenyl)pentyl]glutathione (GIF-0017) as a biochemical probe targeting the ATP-dependent organic anion transporters *GS-X* pumps was synthesized by the reaction of [^{35}S]glutathione and excess 4-(5-bromo)pentylbenzophenone under alkaline conditions, with the radiochemical yield of 24–33% after HPLC purification. Photolysis of the mixture of [^{35}S]GIF-0017 and plasma membrane vesicles prepared from the MRP1 cDNA-transfected LLC-PK1 cells resulted in radio-labeling of a 180-kDa membrane protein. Immunoprecipitation and western blotting using an anti-MRP1 monoclonal antibody confirmed that the [^{35}S]GIF-0017-labeled protein was the *MRP1/GS-X* pump.

Keywords: *MRP1/GS-X* pump; Photoaffinity Labeling; [^{35}S]GIF-0017

The *GS-X* pump family [1] comprises ATP-binding cassette (ABC) transporters, such as MRP1, cMOAT (MRP2), YCF1, and AtMRP identified in human, rat, yeast and plant, respectively [2–11]. Accumulating evidence strongly suggests that these ABC transporters play physiologically important roles in detoxification, inflammation, oxidative stress, and tumor drug resistance. While the *GS-X* pumps transport a wide range of organic anions, glutathione *S*-conjugates are preferential substrates [12]. We have recently demonstrated that the expression of a human *GS-X* pump encoded by the MRP1 gene is closely linked with cellular glutathione (GSH) metabolism and is induced by cisplatin, nitrosoureas, heavy metals

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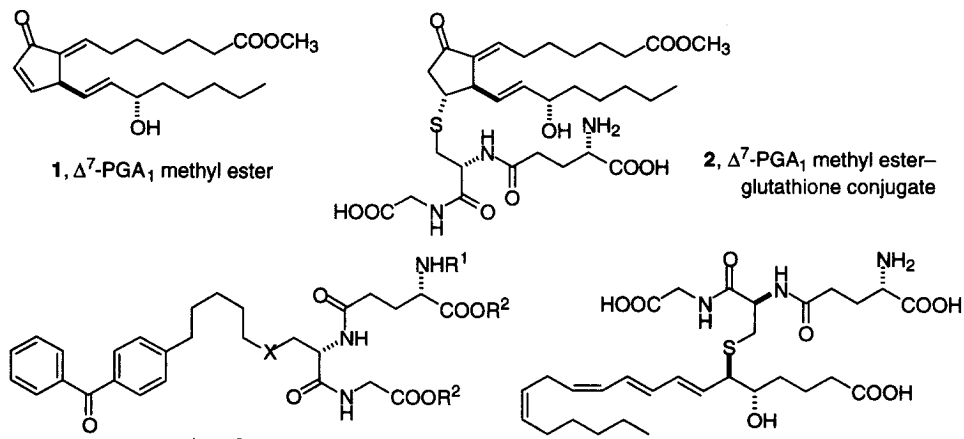
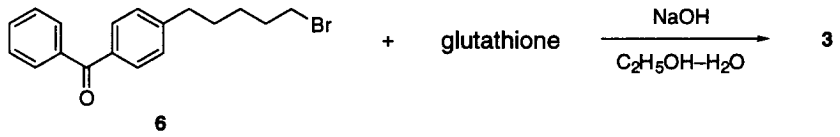
and oxidative stress [13–15]. Coordinately enhanced expression of *MRP1/GS-X* pump and γ-glutamylcysteine synthetase has been found in the cisplatin-resistant human leukemia HL-60 (HL-60/R-CP) cells [13] as well as in colorectal cancer of patients [16]. In a recent study using the HL-60/R-CP cells [17], we have proven that the *MRP1/GS-X* pump participates in GSH-associated cellular resistance to antitumor prostaglandin Δ^7-PGA_1 methyl ester (**1**) [18,19] as supported by the following observations: (1) significant resistance of HL-60/R-CP cells to **1**; (2) facile reaction of PG **1** with glutathione (GSH), a major intracellular thiol constitute, to form a PG–glutathione conjugate **2**; (3) actual ATP-dependent transport of **2** by the *MRP1/GS-X* pump in inside-out vesicles prepared from the resistant cells [17,20–25]. In order to have further insight into the role of the *MRP1/GS-X* pump for the molecular mechanism of cellular drug resistance, we designed and synthesized *S*-[5-(4-benzoylphenyl)pentyl]glutathione (GIF-0017, **3**) as a *GS-X* pump-targeting biochemical probe [26]. Actually, **3** competitively inhibited the *MRP1/GS-X* pump mediated transport of **2** and LTC_4 (**4**) in a dose-dependent manner with IC_{50} values of 0.52 and 0.40 μM, respectively [17,26]. This result suggests that **3** is a good substrate for the *MRP1/GS-X* pump and shares the same binding site of the *GS-X* pump protein with LTC_4 and **2**. Furthermore, **3** was designed as a photoaffinity labeling probe where its benzophenone part plays as a photoactivatable function [27]. This paper describes the synthesis of the corresponding ^{35}S-incorporated radioligand **5** and its validity for photoaffinity labeling of the *MRP1/GS-X* pump protein.



Non-radioactive GIF-0017 (**3**) was synthesized by the usual S_N2 reaction of 4-(5-bromo)pentylbenzophenone (**6**) and an equivalent of glutathione under alkaline conditions as

reported previously (Scheme 1) [26]. However, this method is not applicable to the synthesis of radioactive [^{35}S]GIF-0017 (**5**), which is due to extremely small amount of commercially available [^{35}S]glutathione (ca. 0.3–0.6 nmol). Even if the reaction volume is diminished to a few microliter (practically a lower limit), the concentration of [^{35}S]glutathione reaches no more than 1×10^{-4} M, and the equimolar S_N2 reaction is presumed to be very slow under such conditions. Thus, we examined the reaction conditions suitable for the synthesis of radioactive **5** by cold experiments.

Scheme 1



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First, we decided to increase the total quantity of glutathione to 3 nmol by adding non-radioactive glutathione for acceleration of the reaction, because we judged by simple calculation that the radioactivity of ^{35}S-labeled GIF-0017 containing non-radioactive **3** available by the reaction under such conditions should keep an enough level for detection in the photoaffinity labeling experiment. Since [^{35}S]glutathione is commercially available as a 10 mM aqueous dithiothreitol (DTT) solution, we prepared a solution of 10 mM aqueous DTT (125 μL) containing 3 nmol of cold glutathione (24 μM) to simulate the hot reaction. The solution was extracted twice with ethyl acetate to remove most of DTT, and the water layer was lyophilized to dryness. The residual glutathione was reacted with **6** in the presence of alkali under various conditions. Our initial attempt with the use of slightly excess amounts of sodium hydroxide and **6**, analogous to the preparation of non-radioactive **3**, resulted in incomplete conversion, as estimated. Augmentation of both the bromide and sodium hydroxide accelerated the reaction and increased the conjugate formation. The use of ca. 60-fold bromide was, however, an upper limit because of the solubility problem. During the reaction, we observed a gradual consumption of the product with the time after the reaction reached to the plateau. This is presumably due to further alkylation of the carboxylic moieties of the glutathione residue in the conjugate. Another co-solvents such as dimethylsulfoxide and dimethylformamide instead of ethanol did not improve the reaction. Based on these observations, we set up the following optimum reaction conditions. The lyophilized glutathione was treated with 60-fold amount of **6** and 200-fold sodium hydroxide in 24 μL of water–ethanol (1 : 3) at 40 °C for 2 h. HPLC analysis of the reaction mixture indicated the formation of **3** in 75% yield (not isolated).

According to the above procedure, [^{35}S]*S*-[5-(4-benzoylphenyl)pentyl]glutathione, [^{35}S]GIF-0017 (**5**), was synthesized as described below. Radioactive **5**, which contains ca.

10-fold excess non-radioactive **3**, was obtained in 24–33% radiochemical yields, based on the initial activity of [^{35}S]glutathione after HPLC separation, with the radioactivity of 60–85 μCi. ^{35}S-incorporated GIF-0017 thus obtained was used for photoaffinity labeling of the *MRP1/GS-X* pump. Thus, **5** incubated with the plasma membrane vesicles prepared from MRP-transfected LLC-PK1 cells [28,29] was photolyzed with a 100W mercury lamp at 0 °C for 5 min. Autoradiography after SDS-PAGE resolution indicated a specific label of a polypeptide with an apparent molecular mass of 180 kDa (Mr) (Figure 1A). The labeled band was completely consistent with that stained with an anti-MRP monoclonal antibody in the western blotting [30,31] (Figure 1B), confirming that **5** unambiguously labeled the *MRP1/GS-X* pump [32–37]. These results strongly support our hypothesis for the effect of the enhancement of the cellular sensitivity of HL-60/R-CP cells to anticancer Δ^7-PGA$_1$ methyl ester (**1**) by GIF-0019 (**7**), an esterified derivative of **3** designed as a cell membrane-permeable *GS-X* pump inhibitor: The inhibitor **7** penetrates the membrane to enter cells and subsequently undergoes hydrolysis catalyzed by esterase to form **3**, which blocks the transport of the GS-PG conjugate **2** through the *MRP1/GS-X* pump by directly interacting with the *GS-X* pump protein, resulting in the accumulation of PGs in the cell to realize the efficient supply of the anti-tumor PG (**1**) into nuclei (Figure 2) [17,25,26].

In conclusion, we demonstrated the synthesis of **5** and its successful use for photoaffinity labeling of the *MRP1/GS-X* pump. Since the benzophenone photophore in **5** is a ubiquitous and stable organic function and is characterized by the outstanding ability of mild reversible photoactivation without destruction of the photophore different from another azide- and diazirine-furnished photoaffinity probes [27,38], **5** is expected to be a versatile biochemical probe not only for the analysis of ligand binding domain of the *GS-X* pump but also for related studies including pharmacokinetics, drug metabolism, and tissue mapping of the pump, etc. Further progress will be described in due course.

Synthesis of 5

A solution of [^{35}S]glutathione (NENTM Life Science Products Inc., 261 μCi) in 10 mM aqueous DTT (140 μL) placed in a 1-mL test tube was diluted with 50 μM solution of non-radioactive glutathione in 10 mM aqueous DTT (50 μL, 2.5 nmol). The mixture was extracted with ethyl acetate (0.25 mL × 2) and the water layer was lyophilized to dryness. To the residue was added aqueous sodium hydroxide (100 mM, 6.0 μL, 0.60 μmol) followed by bromide **6** dissolved in ethanol (10 mM, 18 μL, 0.18 μmol). The solution was transferred to a 0.2-mL test tube and sealed with a plastic cap. The mixture was heated at 40 °C for 2 h in the sand-bath, and then allowed to cool to ambient temperature. After centrifugation, the mixture was subjected to HPLC using a polymer C18 column (YMC-Pack PolymerC18, 4.6 mm × 150 mm) eluted with 50% aqueous methanol. The fraction containing **5** and non-radioactive **3**

(1.14 mL) indicated a radioactivity of 85 μCi, which corresponded to 33% radiochemical yield (0.89 μM solution as total glutathione conjugate).

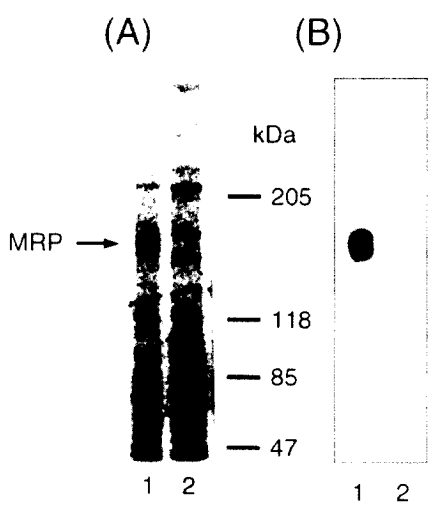

Figure 1. Specific photolabeling of *MRP1/GS-X* pump polypeptide (180 kDa) by [^{35}S]GIF-0017. (A) Autoradiography. (B) Western blot analysis with anti-MRP monoclonal antibody. Lanes 1 and 2 show MRP-transfected and untransfected LLC-PK1 cells, respectively.

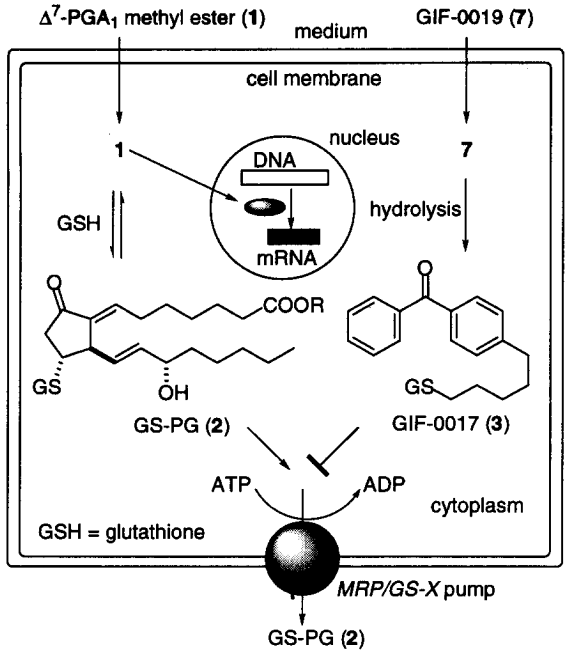

Figure 2. Molecular mechanism underlying the sensitization of HL-60/R-CP cells to Δ^7-PGA$_1$ methyl ester (**1**) by co-incubation with the *GS-X* pump inhibitor GIF-0019 (**7**).

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