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COMMUNICATION

A mechanism-based fluorescent probe for labeling O^6-methylguanine-DNA methyltransferase in live cells†

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A mechanism-based small molecular fluorescent probe has been developed to label active O^6-methylguanine-DNA methyltransferase in live cells.

Many alkylating antitumor agents, such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and temozolomide, provoke cytotoxic effects by targeting the O^6-position of guanine in DNA.[1] However, the therapeutic efficacy of these drugs can be significantly attenuated by the repair capacity of O^6-methylguanine-DNA methyltransferase (MGMT), which restores DNA with O^6-alkylguanine lesions by transferring the alkyl groups to an internal cysteine residue *via* an irreversible and stoichiometric reaction.[2] A number of *in vitro* and *in vivo* studies have established a negatively correlated relationship between the levels of MGMT and the sensitivity to guanine O^6-targeting drugs.[3] MGMT is therefore considered a crucial biomarker for individual susceptibility to alkylating agents. Apart from its importance in clinical resistance to alkylating drugs, MGMT is also of great interest because, although it is not essential for DNA replication or cell survival, it is expressed in normal cells at levels that far exceed its need to repair endogenous DNA lesions and the underlying role remains an enigma.[4]

MGMT is highly variably expressed in tumors.[5] There are various methods available for MGMT determination, including the complex and laborious assays based on radioactive DNA substrates, and the measurement of immunoblots by anti-MGMT antibodies, *etc*.[6] However, all these techniques are limited to cell lysates and require cell homogenation, a process that disrupts cellular organization and may destroy the balance between active MGMT and its endogenous inactivated state.[7] Thus, the results obtained may not represent the actual situation of MGMT in a living cell. Moreover, the employment of radioactive isotopes in these assays requires more care in handling in a laboratory setting. A simple, nonradioactive and cell permeable probe to detect MGMT in live cells would prove more relevant for the research on the protein's role in pathophysiology and physiology.

In our continuous work on the development of specific fluorescent probes for biomedical research,[8] we report herein the design, synthesis and biological application of a mechanism-based probe, **1**, which enables direct live cell labeling and imaging of MGMT as well as subsequent fluorogenic analysis. Probe **1** was designed on the basis of O^6-benzylguanine (O^6-BG, **2**) (Fig. 1), a potent MGMT substrate which has been shown unequivocally to inactivate MGMT effectively, resulting in the formation of *S*-benzylcysteine in the protein and the stoichiometric production of guanine.[9] Based on this inactivation mechanism, probe **1** was designed by attaching a fluorescent moiety onto the '*meta*' position of the benzyl ring in **2**, a position which has been revealed to tolerate even quite large substituents.[10] A poly(ethylene glycol) (PEG) chain was employed to link the fluorophore with the O^6-benzylguanine scaffold. This chain acts to increase the probe's solubility and decreases its steric hindrance which may exclude access to the active site of MGMT. The well known 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene (BODIPY) was selected as the fluorescent moiety because of its

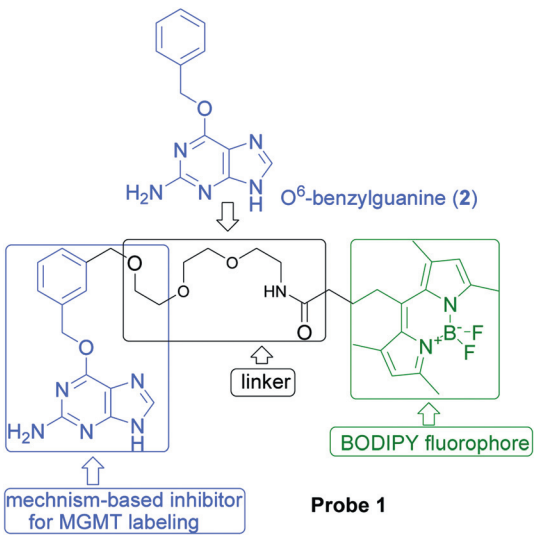

Fig. 1 Structure of probe **1** and O^6-benzylguanine (**2**). The structure template of **1** consists of the O^6-BG moiety for binding and labeling MGMT, a PEG linker to minimize the steric hindrance, and a BODIPY fluorophore for visualization and fluorogenic analysis of the labeled protein.

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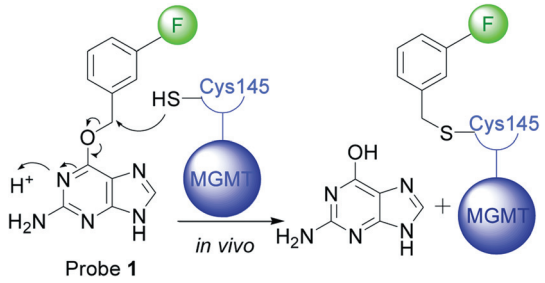
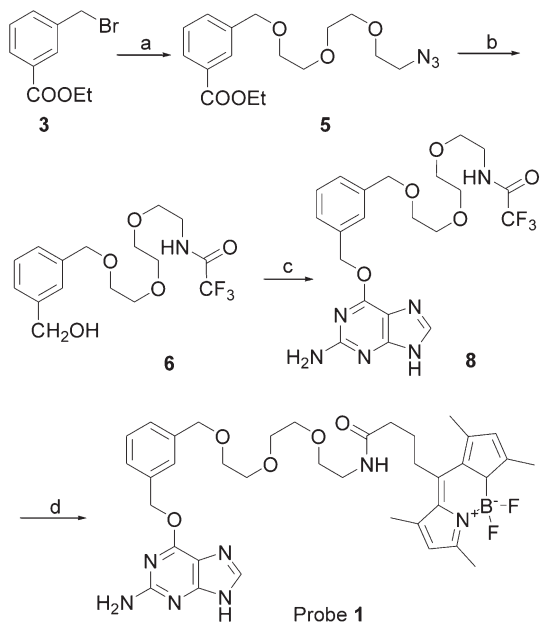


Fig. 2 Schematic representation of MGMT labeling by probe **1**. The probe acts as a substrate to inactivate MGMT and labels the protein with the benzyl group bearing a fluorophore (F).



Scheme 1 Synthesis of the mechanism-based probe **1**. Reagents and conditions: (a) **4**, NaH, dry THF, 0 °C–r.t., 58%; (b) (i) $LiAlH_4$, dry THF, reflux, 2 h; (ii) CF_3COOEt, Et_3N, anhydrous EtOH, r.t., 5 h, 87% in two steps; (c) ^tBuOK, **7**, DMF, 0 °C–r.t., 3 h, 58%; (d) (i) K_2CO_3, MeOH, 60 °C, 3 h; (ii) Et_3N, **9**, anhydrous MeOH, 91% in two steps.

many attractive spectral characteristics and its hydrophobic nature.[11] Probe **1** is expected to be a pseudosubstrate of MGMT. It should inactivate MGMT in the same way as compound **2**, and label MGMT, taking advantage of the transfer of its fluorescent benzyl residue to the protein's active site (Fig. 2).

Preparation of probe **1** is outlined in Scheme 1. Nucleophilic substitution of ethyl 3-(bromomethyl)benzoate (**3**) with 2-(2-(2-azidoethoxy)ethoxy)-ethanol (**4**) yielded **5**, which was reduced with $LiAlH_4$, followed by protection of the amine group as the trifluoroacetate **6**. The potassium alkoxide of **6** was reacted with 1-(2-amino-9*H*-purin-6-yl)-1-methylpyrrolidinium chloride (**7**)[12] to furnish the crucial intermediate **8**. Deprotection of **8** and subsequent acylation with the activated ester **9**, *N*-hydroxysuccinimidyl-4-(4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene-8-yl)-butyric acid,[13] yielded probe **1**.

To demonstrate the feasibility of **1** as a sensitive probe for MGMT in live cells, it was first evaluated for its ability to

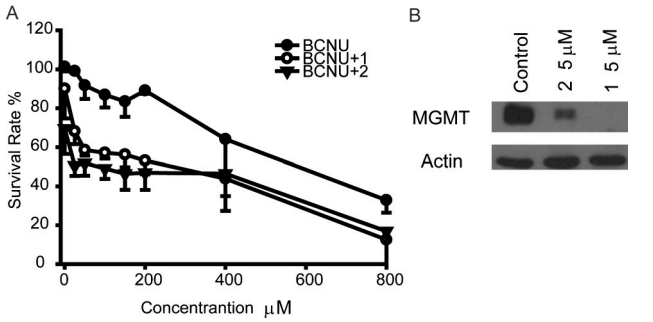


Fig. 3 Probe **1** is a substrate of MGMT. Exposing HT 29 cells to probe **1** or **2** at 5 μM for 2 hours prior to the addition of 0–800 μM BCNU resulted in a dramatic decrease of cell survival, implying the potent activity of **1** to inactivate cellular MGMT (A). Treating HT29 cells with probe **1** or **2** at 5 μM for 24 hours led to the degradation of MGMT, while the non-treatment control remained to express MGMT at a high level (B). All experiments were run in triplates.

inactivate MGMT. Adapted from the method reported by Pauly *et al.*,[12] a methylthiazolyldiphenyl-tetrazolium-bromide (MTT) colorimetric assay was carried out to determine the inhibitory effect of **1** on MGMT by testing the sensitization of HT 29 cells to killing by BCNU. As shown in Fig. 3A, probe **1** alone had no cytotoxicity at 5 μM, while it significantly improved the efficacy of BCNU. This sensitization illustrated that probe **1** inactivated cellular MGMT effectively and counteracted the cells' resistance to BCNU, an effect similar to that of O^6-BG (**2**). To further verify this conclusion, MGMT expression levels in HT 29 cells were examined before/after treatment with probe **1** using an immunoblotting technique with anti-MGMT. It turned out that exposure of HT 29 cells to probe **1** at a concentration of 5 μM for 24 hours led to a significant decline of MGMT level (Fig. 3B), which is in agreement with the previous report that inactivated MGMT was degraded slowly *via* the ubiquitin proteolytic pathway.[7] In summary, these data suggested that probe **1** retained MGMT substrate properties, confirming its validity as a mechanism-based probe to label MGMT in live cells.

Probe **1** was then tested for its capacity to label MGMT in live cells. HT 29 or HeLa S3 cells were incubated with probe **1** (5 μM) for 20 min. At the end of the incubation period, cells were washed with phosphate buffered saline (PBS) twice and subsequently processed for microscopy analysis using a Leica DMI4000B fluorescence microscope. An incubation time of 20 min was established based on the observation that intracellular fluorescence was visible after an incubation time of 5 min and reached the saturation point at around 20 min. The concentration was also determined based on dose–response studies, which showed that intracellular fluorescent labeling was detectable using concentrations of 2–10 μM of probe **1**, but a concentration of 5 μM was required to obtain consistent and reproducible labeling. Under these conditions, cells appeared to be stained mainly in the cytoplasm (Fig. 4A, a, c), in agreement with a previous report on the cytoplasmic localization of MGMT in HeLa S3 cells.[14]

Competition experiments were also performed in parallel to test the specificity of probe **1**. For this purpose, cells were pre-incubated with O^6-BG (50 μM) for 30 min, and then were

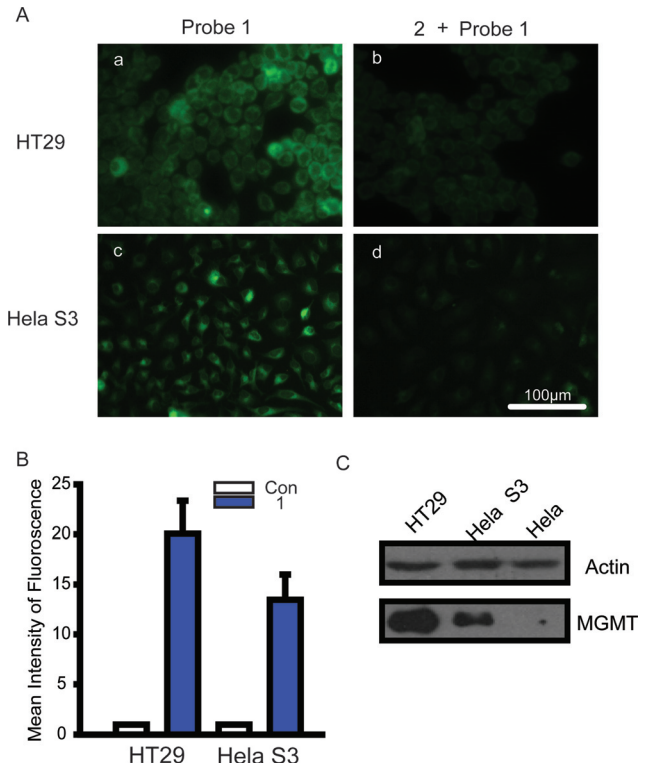


Fig. 4 (A) Fluorescent labeling studies of MGMT in HT 29 and Hela S3 cells by probe **1** with or without **2** as a competing substrate. Pretreatment of cells with **2** (b, d) significantly eliminated the fluorescent intensity compared with no pretreatment (a, c). Cells were detected after excitation (ex) at 488 nm. Scale bar represents 100 μm; (B) quantification of the fluorescent labeling events. HT 29 and Hela S3 cells were treated with 5 μM **1** for 20 min, followed by twice of wash with PBS. The mean fluorescence intensity was then recorded (arbitary units, *A.U.*) and expressed as means ± SEM from three independent experiments. The significant levels (**) represent $p < 0.005$; (C) HT 29, Hela S3, and Hela cells express MGMT at different levels, as shown by immunoblot assay with anti-MGMT antibody.

stained with probe **1** (5 μM). As expected, the fluorescent staining was significantly decreased presumably due to the pre-occupation of the active site of MGMT by **2**, demonstrating the specificity of the interaction between probe **1** and MGMT (Fig. 4A, b, d).

The above labeling events were also quantified by recording the mean fluorescence intensity. It turned out that HT 29 cells exhibited a stronger fluorescence intensity compared with Hela S3 cells (Fig. 4B), in agreement with the results that intact HT 29 cells contain a higher concentration of MGMT as determined by immunoblot assay with anti-MGMT antibody (Fig. 4C). This verifies the potential of probe **1** as a tool to detect active MGMT quantitatively in live cells.

In conclusion, we have developed a mechanism-based small molecular probe for the fluorogenic analysis of MGMT in live cells. The probe, designed based on a mechanism-based inhibitor of MGMT and the structure–activity relationship thereof, shows a great deal of significance. First, it retains MGMT's substrate properties. Second, it is easily cell permeable and specifically labels active MGMT but not its inactivated counterpart in intact mammalian cells. Furthermore, the labeling of cells may be quantitatively analyzed. Therefore, probe **1** represents a valuable tool for live cell study of the location, function, and quantification of MGMT in both the healthy and diseased state. Further studies are now planned to develop an MGMT assay based on probe **1**.

Acknowledgements

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