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Measurement of O^6-alkylguanine-DNA alkyltransferase activity in tumour cells using stable isotope dilution HPLC-ESI–MS/MS

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ABSTRACT

The repair of DNA mediated by O^6-alkylguanine-DNA alkyltransferase (AGT) provides protection against DNA damage from endogenous or exogenous alkylation of the O^6 position of guanine. However, this repair acts as a double-edged sword in cancer treatment, as it not only protects normal cells from chemotherapy-associated toxicities, but also results in cancer cell resistance to guanine O^6-alkylating antitumour agents. Thus, AGT plays an important role in predicting the individual susceptibility to guanine O^6-alkylating carcinogens and chemotherapies. Accordingly, it is necessary to establish a quantitative method for determining AGT activity with high accuracy, sensitivity and practicality. Here, we describe a novel non-radioactive method for measuring AGT activity using stable isotope dilution high-performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC-ESI–MS/MS). This method is based on the irreversibility of the removal of the O^6-alkyl group from guanine by AGT and on the high affinity of O^6-benzylguanine (O^6-BG) as an AGT substrate. HPLC-ESI–MS/MS was used to measure the AGT activities in cell protein extracts from eight tumour lines, demonstrating that AGT activity was quite variable among different cell lines, ranging from nondetectable to 1021 fmol/mg protein. The experiments performed in intact tumour cells yielded similar results but exhibited slightly higher activities than those observed in cell protein extracts. The accuracy of this method was confirmed by an examination of AGT expression levels using western blotting analysis. To our knowledge, this method is the first mass spectrometry-based AGT activity assay, and will likely provide assistance in the screening of cancer risk or the application of chemotherapies.

1. Introduction

A large number of endogenous methylating agents (e.g., S-adenosylmethionine), environmental carcinogens (e.g., N-nitrosodimethylamine) and antitumour alkylating agents, such as methylating agents (e.g., temozolomide, dacarbazine and procarbazine) and chloroethylating agents (e.g., carmustine, lomustine and laromustine), can attack the O^6 position of guanine in the DNA duplex to form O^6-alkylguanine, which is considered as a cytotoxic, mutagenic and carcinogenic lesion [1–4]. If not repaired correctly, these lesions can induce cell apoptosis or malignant transformation [1,4–6]. O^6-alkylguanine-DNA alkyltransferase (AGT), also called O^6-methylguanine-DNA methyltransferase (MGMT), is a unique DNA repair protein that repairs O^6-alkylguanine lesions in DNA by transferring the alkyl lesion groups from the O^6 position of guanine to its own active centre cysteine 145 (Cys145) to restore normal DNA structure (Fig. 1). This action inactivates the protein and exhausts the repair capacity until the de novo generation of new enzyme molecules. After accepting the alkyl group, the inac-

Abbreviations: AGT, O^6-alkylguanine-DNA alkyltransferase; HPLC-ESI–MS/MS, high-performance liquid chromatography electrospray ionization tandem mass spectrometry; O^6-BG, O^6-benzylguanine; Cys145, Cysteine 145; O^6-MG, O^6-methylguanine; [^{3}H]MNU, N-[^{3}H]-methyl-N-nitrosourea; UV, ultraviolet; NMR, nuclear magnetic resonance; MEM-EBSS, Eagle's minimal essential medium with Earle's balanced salt solution; FBS, foetal bovine serum; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; CID, collision induced dissociation; SRM, selected reaction monitoring; SDS-PAGE, SDS polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBST, phosphate buffered saline solution containing 0.05% Tween 20; SPSS, Statistical Package for the Social Sciences software; SD, standard deviation; LOD, limit of detection; LOQ, limit of quantitation; RSD, relative standard deviation; O^6-4-BTG, O^6-(4-bromothenyl)guanine; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

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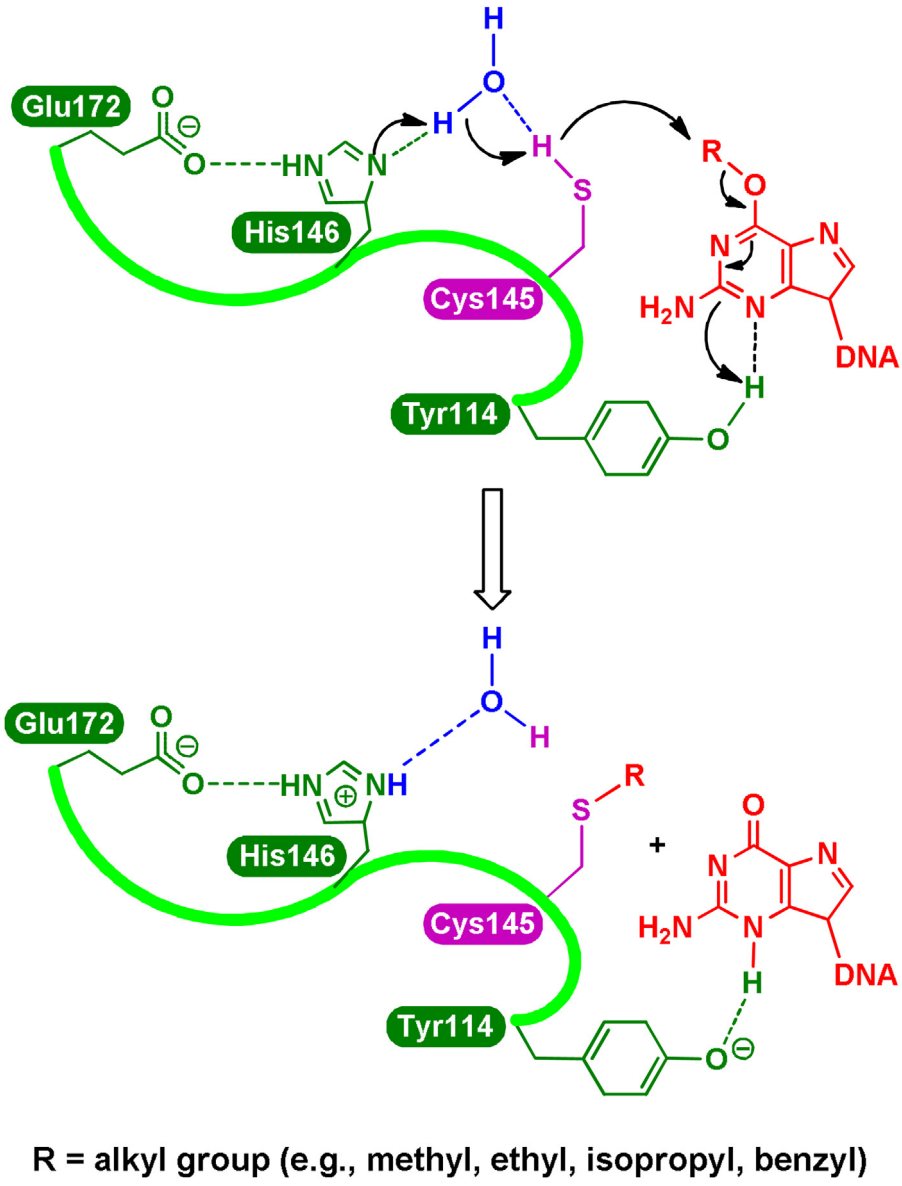

Fig. 1. Proposed mechanism for AGT-mediated repair of O^6-alkylguanine.

tivated protein is degraded rapidly by a ubiquitination-dependent proteolysis pathway [7–10]. Thus, AGT is a suicide enzyme and acts only once. In normal cells, AGT-mediated repair protects cells from the cytotoxic lesions produced by environmental carcinogens or chemotherapeutic agents [1,11], whereas in tumour cells, AGT-mediated repair is likely to result in drug resistance thereby reducing the anticancer efficacy of chemotherapies that alkylate DNA at the O^6 position of guanine [2,4]. Previous studies indicated that there was an inverse relationship between the content of AGT and the sensitivity of cells to guanine O^6-alkylating agents [12–16]. It is worth noting that cellular AGT content is highly variable in normal and tumour tissues [13,17]. In particular, AGT is specifically expressed at high levels in ovarian and breast tumours and at relatively low levels in some brain tumours [17]. A lower AGT content in tumour cells than in normal cells is a prerequisite for the selectivity of guanine O^6-alkylating agents towards tumour tissues [15]. Therefore, AGT content represents an important factor in estimating individual susceptibility to guanine O^6-alkylating carcinogens and antitumor drugs [1,18,19]. To better understand the role of AGT in cancer prevention or chemotherapy application, it is necessary to establish a practical method for determining the activity of AGT. The classical method for AGT activity assessment, which uses DNA containing [^{3}H]-labelled O^6-methylguanine (O^6-MG) induced by N-[^{3}H]-methyl-N-nitrosourea ([^{3}H]MNU) as a substrate, requires a multi-step procedure including incubation of cell extracts with the DNA substrate, DNA purification followed by hydrolysis, and measurement of the removal of the [^{3}H]-labelled methyl or of the radioactivity transfer to AGT [20,21]. Another method utilized a double-stranded oligonucleotide containing O^6-MG in a restriction site labelled with a fluorescent group or radioactively end-labelled with ^{32}P or ^{35}S [22–24]. Recently, Nagel et al. [25] and Georgiadis et al. [26] developed a nonradioactive ELISA-type method for determining the AGT activities in human normal or tumour tissues. Ishiguro et al. [27] established a relatively simple method for AGT activity assay using [^{3}H]-labelled O^6-BG as substrate. However, the above methods are limited in clinical application because of their relatively complicated procedure, requirement of radioactive materials, or somewhat low sensitivity and specificity. In this study, we report a novel non-radioactive method for determining the AGT activity in