Drug Discovery Hot Paper

International Edition: DOI: 10.1002/anie.201511301
German Edition: DOI: 10.1002/ange.201511301

Chemoproteomics-Enabled Discovery of a Potent and Selective Inhibitor of the DNA Repair Protein MGMT

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Abstract: *We present a novel chemical scaffold for cysteine-reactive covalent inhibitors. Chloromethyl triazoles (CMTs) are readily accessed in only two chemical steps, thus enabling the rapid optimization of the pharmacological properties of these inhibitors. We demonstrate the tunability of the CMTs towards a specific biological target by synthesizing AA-CW236 as the first potent non-pseudosubstrate inhibitor of the O^6-alkylguanine DNA methyltransferase (MGMT), a protein of major clinical significance for the treatment of several severe cancer forms. Using quantitative proteomics profiling techniques, we show that AA-CW236 exhibits a high degree of selectivity towards MGMT. Finally, we validate the effectiveness of our MGMT inhibitor in combination with the DNA alkylating drug temozolomide in breast and colon cancer cells by fluorescence imaging and a cell-viability assay. Our results may open a new avenue towards the development of a clinically approved MGMT inhibitor.*

DNA is a chemically vulnerable molecule, and our cells have developed sophisticated repair mechanisms to maintain the integrity and stability of DNA.[1] The DNA repair protein O^6-methylguanine-DNA-methyltransferase (MGMT) transforms the naturally occurring DNA lesion O^6-methylguanine back into guanine.[2] During this transformation, the O^6-methyl group is transferred to the active-site cysteine, irreversibly inactivating MGMT. This attribute of MGMT has been exploited for designing a self-labeling protein tag.[3] Unfortunately, MGMT also repairs damage caused by DNA alkylating agents in cancer cells.[4] For example, MGMT activity levels in brain tumors can vary by a factor of up to 300, and there is a strong correlation between high MGMT levels in patients and failed response to chemotherapy.[5] Pseudosubstrate inhibitors, such as O^6-benzylguanine and O^6-(4-bromothienyl)guanine (lomeguatrib), irreversibly alkylate the active-site cysteine in MGMT and have thus been tested in clinical trials in combination with DNA alkylating agents.[6] Phase I trials based on DNA alkylating drug temozolomide/lomeguatrib combinations were successful and attested effective depletion of MGMT activity and higher formation of the desired O^6-MeG adducts in human patients with various cancer forms.[7] Unfortunately, DNA alkylating agents such as temozolomide also cause severe myelosuppression in patients, and this side effect is even increased by the co-administration of MGMT pseudosubstrates.[8] Interestingly, the level of myelosuppression varies markedly between the different pseudosubstrates, making us optimistic that this serious side effect can at least be improved with new MGMT inhibitors.[9] Paradoxically, the reaction between the highly dosed MGMT pseudosubstrates and the protein itself creates free guanine base as a side product that can be utilized by the cancer cells to synthesize new DNA, which is exactly the opposite effect of what many anticancer agents aim to achieve.[10] Thus, we believe that there is a need for new, non-O^6-alkylguanine-based MGMT inhibitors. Herein, we present chloromethyl triazoles (CMTs) as a new, tunable cysteine-reactive chemical scaffold that can be accessed in only two chemical steps, enabling rapid preparation of a large series of compounds and also streamlining the optimization of inhibitor potency and selectivity. By applying methods from biochemistry, cell biology, and quantitative proteomics, we discover and evaluate AA-CW236 as the first potent and selective non-pseudosubstrate inhibitor of MGMT.

Covalent inhibitors currently enjoy a renaissance of popularity in pharmaceutical research.[11] The proposal of chloromethyl triazoles as a scaffold for cysteine-reactive covalent inhibitors (Figure 1 A) was inspired by the shape of N-heterocyclic carbene ligands, which are widely used in catalysis,[12] where both nitrogen substituents are sterically protecting the reactive metal center by pointing towards it.

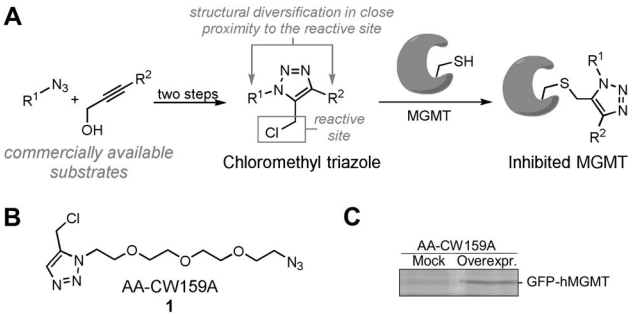

Figure 1. Testing the reactivity of a clickable chloromethyl triazole probe. A) A general reaction scheme showing the concept and the synthetic accessibility of CMTs and the expected mechanism of MGMT inhibition. B) Chemical structure of the clickable CMT probe **1** (AA-CW159A). C) Gel-based fluorescence labeling of mock-transfected MCF7 lysate and lysate with overexpressed GFP-hMGMT (the full-length gel image is shown in Figure S2).

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Supporting information for this article (synthesis of compounds, cell culture, bioassays, labeling and sample preparation for proteomics, and mass spectrometry) is available on the WWW under http://dx.doi.org/10.1002/anie.201511301.

We hypothesized that owing to the close proximity to the chloromethyl group, groups R^1 and R^2 should strongly influence both the chemical reactivity and selectivity of the inhibitors.

To test whether CMTs covalently react with proteomic cysteines, we synthesized the clickable probe AA-CW159A (**1**; Figure 1B). We then treated breast-cancer-derived MCF7 cellular lysates with probe **1** at 10 μM concentration, which were then reacted with TAMRA alkyne through copper(I)-catalyzed alkyne–azide cycloaddition (CuAAC), separated by SDS-PAGE, and visualized by in-gel fluorescence scanning. Multiple bands were detectable, and the fluorescence labeling was completely abolished by pretreatment with the broad-profile cysteine-reactive probe iodoacetamide, indicating that **1** shows a strong preference for the labeling of cysteines over other nucleophilic amino acids (Supporting Information, Figure S1). We next performed a proteomics experiment to explore whether MGMT can be labeled and enriched by probe **1**. Briefly, MCF7 cell lysates were treated with 10 μM of probe **1**, clicked to biotin alkyne, enriched over streptavidin beads, and analyzed by LC-MS/MS. MGMT was identified as a target of probe **1** along with many other proteins (Table S1). We confirmed that the CMT model probe **1** is indeed capable of covalently binding to MGMT by overexpressing the GFP-tagged human MGMT in MCF7 cells and performing gel-based fluorescence labeling with **1**. A strong new fluorescent band at 52 kDa indicates the formation of a covalent bond between the CMT probe and MGMT (Figures 1C, S2).

Encouraged by these results, we prepared a small collection of 1,4-substituted CMTs **2**–**19** (Figure 2A). Starting from commercially available alkyl azides and substituted propargylic alcohols, 5-hydroxymethyl triazoles were accessed by thermal azide–alkyne cycloaddition. Alternatively, triazoles could also be efficiently prepared from more temperature-sensitive substrates by ruthenium-catalyzed azide–alkyne cycloaddition[13] (RuAAC). With both methods, the 1,4-substituted isomer was generally obtained as the major product and separated from the 1,5-substituted isomer by flash chromatography. Finally, the hydroxymethyl triazoles were converted into the corresponding chlorides by mesylation and nucleophilic substitution with tetrabutylammonium chloride in one-pot fashion. The 1,4-substitution of the CMTs was unambiguously confirmed by X-ray structural analysis (for five CMTs) and by HMBC NMR analysis of the alcohol precursors. In parallel, we established a gel-based competitive proteomics profiling experiment to identify potent MGMT binders. Briefly, cellular lysates with overexpressed hMGMT were treated with compounds at 200 nM concentration, followed by probe **1** (Figures 2A, B, and S3). Whereas most of the CMTs did not compete the fluorescence labeling of MGMT, $>70\%$ competition was observed with triazole **14**. Some activity was also detectable with CMTs **17** and **18**. Finally, the best competition of MGMT fluorescence labeling (88%) was achieved with compound **19** (AA-CW236), which includes both active structural elements, the 3,5-dimethylisoxazole group and the 4-(trifluoromethoxy)phenyl substituent (Figures 2B and S4).

As hMGMT contains five different cysteines, it was important to ascertain that the CMTs indeed react with the

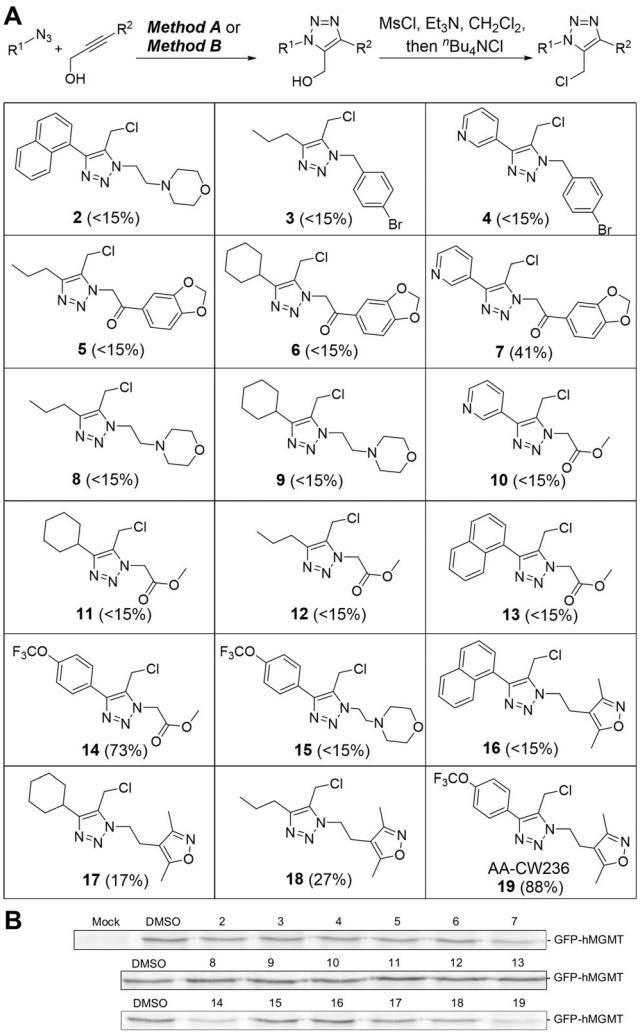

Figure 2. Synthesis and gel-based screening of 1,4-substituted CMTs. A) General synthetic route to 1,4-substituted CMTs and chemical structures of CMTs **2**–**19** (method A: PhMe, 125 °C; method B: Cp*Ru-$(PPh_3)_2Cl$ (2 mol%), PhMe, 80 °C). Values in parentheses indicate the competition (in %) of GFP-hMGMT labeling with probe **1** (AA-CW159A). All compounds were screened at a concentration of 200 nM. B) Images from the gel-based competitive screening of CMTs **2**–**19**.

active-site Cys^{145}. In contrast to the overexpressed wild type (WT) protein, the C145A MGMT mutant was not labeled by probe **1**, meaning that CMTs bind exclusively to Cys^{145} (Figure 3A). As AA-CW236 binds to MGMT covalently and irreversibly, we applied the method of Kitz and Wilson to determine the binding constants K_I and k_{inact}.[14] Our kinetics assay relies on measuring the competition of probe **1** and not on substrate turnover and is therefore applicable to non-enzyme proteins such as MGMT. The calculated K_I value of 24 nM demonstrates that AA-CW236 is a highly potent inactivator of human MGMT. For comparison, AA-CW236 is as potent as lomeguatrib and approximately ten times more potent than O^6-benzylguanine (Figure S5). However, we believe that selectivity is more relevant for predicting the clinical potential of covalent inhibitors, which are traditionally considered to be more promiscuous.[11] First, we evaluated the selectivity of AA-CW236 in a gel-based assay using MCF7

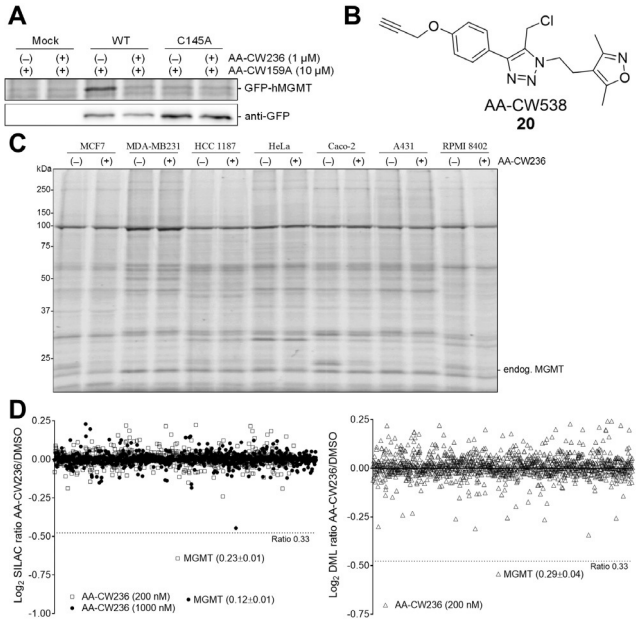

Figure 3. Evaluation of the proteome-wide selectivity of AA-CW236. A) The clickable CMT probe AA-CW159A labels WT GFP-hMGMT, but not the C145A mutant. B) Chemical structure of the clickable CMT probe **20** (AA-CW538). C) Gel-based profiling of the MGMT activity and AA-CW236 selectivity in proteomes of different human cancer cell lines. Proteomes were treated with DMSO or 200 nM AA-CW236 for 1 h followed by 30 μM AA-CW538. D) Evaluation of the AA-CW236 selectivity by quantitative proteomics. Shown are scatter plots with SILAC and DML ratios. Left: SILAC ratios from the competitive experiment with 200 nM ($n=3$) or 1 μM ($n=4$) AA-CW236 treatment followed by 10 μM AA-CW159A. Right: DML ratios from the competitive experiment with 200 nM AA-CW236 ($n=6$) followed by 10 μM IAA alkyne. Ratios ± SEM for MGMT are shown.

lysates with overexpressed hMGMT and the CMT probe **1** (Figure S4). We observed complete competition of MGMT after treatment with 300 nM AA-CW236, whereas no other targets of probe **1** were visibly affected. Whereas the broad-profile probe **1** proved useful for experiments with overexpressed MGMT, it did not allow gel-based detection of native MGMT activity owing to comparably low expression levels of this protein. Therefore, we prepared the expectedly more MGMT-directed probe **20** (AA-CW538), a clickable derivative of the inhibitor AA-CW236 (Scheme S1).

With the new probe in hand, we were finally able to detect the native MGMT activity in various cancer cell lines (Figures 3C, S6). Consistent with the literature,[15] high MGMT activity levels were observed in breast-cancer-derived MCF7 cells. Furthermore, we found very high activity in colon cancer Caco-2 cells and acute lymphoblastic leukemia RPMI 8402 cells. The gel-based assay for profiling the MGMT activity with probe AA-CW538 should also be applicable to clinical samples and represent a significant improvement in terms of convenience and throughput over currently existing radioactive and northern blot based MGMT activity assays.[16] Remarkably, although many other proteins were also labeled by the probe AA-CW538 at 30 μM concentration, only the MGMT band was selectively competed after treatment with 200 nM AA-CW236. Finally, probe **20** also proved useful for measuring the activity of AA-CW236 directly in living MCF7 cells (Figure S7). The gel-based profile indicates that MGMT was completely inhibited after treatment of cells with 100 nM of AA-CW236 for ten hours.

MS-based profiling methods offer a clear advantage over gel-based techniques, because a much higher number of proteins can be detected and quantified in one single experiment.[17] Therefore, we performed a competitive activity-based protein profiling experiment in MCF7 proteomes in combination with quantification by stable isotope labeling of amino acids in culture (SILAC). We used the MGMT inhibitor AA-CW236 at two different concentrations, 200 nM (Table S2) and 1 μM (Table S3), and the broad-profile CMT probe **1** for subsequent proteomic enrichment. Intriguingly, among the combined 1039 quantified proteins and even at 1 μM concentration, only MGMT was found to be greater than 66% competed (SILAC ratio of 0.12 = 88% competition; Figure 3D). Moreover, we conducted an additional competitive proteomics experiment based on the catch-and-release strategy recently reported by us, using iodoacetamide (IAA) alkyne as a general cysteine-reactive enrichment probe,[18] but applying dimethyl labeling (DML) as an alternative method for stable-isotope-based quantification. This method allows selective enrichment and quantification of cysteine-containing and IAA-modified peptides rather than of whole proteins. Again, after treatment with 200 nM AA-CW236, among all 1245 enriched and quantified peptides, only one peptide, which contained the MGMT active-site cysteine, was found to be greater than 66% competed (Figure 3D, Table S4). This is remarkable considering that the human proteome contains thousands of cysteines that are capable of reacting with electrophilic probes.[18,19] In fact, even highly optimized and clinically approved kinase inhibitors bind to numerous off-target cysteines.[20] For comparison, when lomeguatrib (200 nM) was used as a competitor, we observed several other > 66% competed peptides originating from proteins such as the arginine tRNA ligase RARS, guanine nucleotide binding protein GNB2, and dynamin-2 (Figure S8, Table S5).

To understand the molecular basis for the observed potency of AA-CW236 towards hMGMT, we performed covalent docking using a high-resolution X-ray structure of hMGMT (PDB: 1EH6) and the protein-ligand docking program GOLD[21] (Figure 4A). The predicted optimal binding mode suggests a key interaction between the trifluoromethoxy group of AA-CW236 and Tyr^{114} as well as an additional potential interaction between the triazole group and Ser^{159}. Whereas the predicted binding mode for AA-CW236 yielded a GoldScore of 46.0, significantly lower values (35–40) were calculated for the less active derivatives **8**, **9**, and **11**, thus supporting the proposed model. Moreover, to further investigate the role of the CF_3 group in hMGMT binding, we prepared two structurally close derivatives of AA-CW236 that lack this functionality. Subsequent gel-based IC_{50} measurements confirmed that these compounds are indeed significantly less active than AA-CW236 (Figure S9).

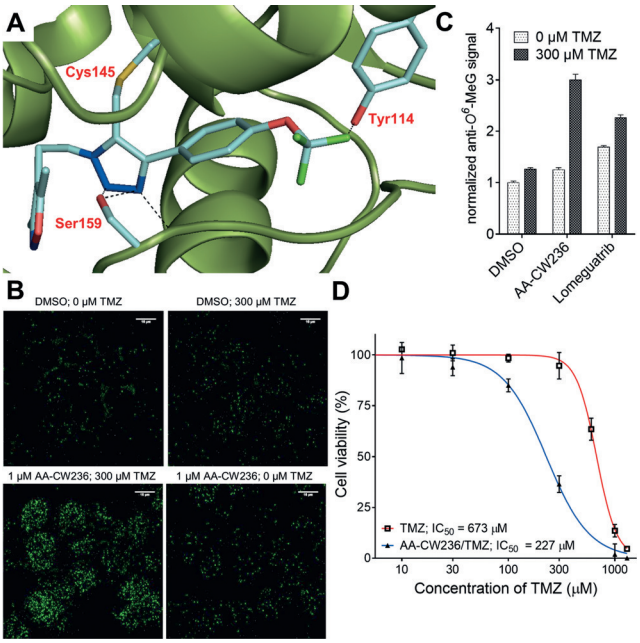

Figure 4. Proposed binding mode and the cancer cell sensitization effect of AA-CW236. A) Docking prediction of AA-CW236 covalently bound to Cys^{145} of human MGMT. B) Detection of guanine O^6-alkylation with anti-O^6-MeG antibody after 12 h treatment with DMSO or AA-CW236 followed or not by the addition of temozolomide (shown are confocal microscopy images; white bars indicate 10 μm). C) Quantification of the guanine O^6-alkylation staining (shown are relative values ± SEM, $n = 3$). The values were normalized against DAPI staining. D) Caco-2 cell viability curve after treatment with various concentrations of TMZ with or without 3 μM AA-CW236 ($n = 3 \pm$ SD).

Finally, we sought to investigate whether the potent inhibition of MGMT by AA-CW236 also leads to increased guanine O^6-alkylation in cancer cells, especially in combination with the drug temozolomide (TMZ). TMZ is currently clinically used as a first-line treatment for glioblastoma multiforme and a second-line option for astrocytoma.[22] Using an O^6-alkylguanine-directed antibody, we established a confocal fluorescence microscopy based method for the direct visualization of nuclear O^6-alkylated guanine in MCF7 cells (Figures 4B, S10). Treatment of the cells with 300 μM TMZ for eight hours resulted only in an approximately 25% increase in O^6-alkylguanine staining. Administration of 1 μM AA-CW236 to MCF7 cells led to a similarly low increase in guanine alkylation. However, when both compounds were added to cells together, we detected a robust threefold increase in O^6-alkylguanine staining (Figures 4C, S10). Intriguingly, the observed sensitization[15] of MCF7 cells was significantly stronger than the one caused by combining TMZ with the best reported[9] MGMT pseudosubstrate lomeguatrib (Figure S10). Furthermore, a sensitization effect was also detected in a cell-viability assay with Caco-2 cells, which display comparably high MGMT activity (Figure 3C) and are resistant to TMZ.[23] Co-treatment with 3 μM AA-CW236 and TMZ significantly reduced the cell viability in comparison to TMZ (Figure 4D) or AA-CW236 (Figure S11) treatments.

In summary, we have presented a novel chemical scaffold for cysteine-reactive covalent inhibitors. Rapid and modular synthesis of structurally diverse chloromethyl triazoles led to the discovery of AA-CW236 as a potent low-nanomolar non-pseudosubstrate inhibitor of MGMT. By using various gel- and MS-based proteomic profiling techniques, we have demonstrated that AA-CW236 is active in cells and exhibits a high degree of selectivity towards MGMT. Furthermore, a strong sensitizing effect was observed in MCF7 and Caco-2 cells when AA-CW236 was co-administrated with the DN alkylating agent TMZ. Finally, while there is no guarantee that this particular compound will be successful in clinical studies, we firmly believe that the results presented herein justify additional efforts towards the identification of improved MGMT inhibitors.

Acknowledgements

The University of Geneva, the Swiss National Science Foundation, and the NCCR Chemical Biology are acknowledged for financial support.

Keywords: activity-based protein profiling · drug discovery · mass spectrometry · MGMT · proteomics

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 2911–2915
Angew. Chem. **2016**, *128*, 2964–2968

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Received: December 6, 2015
Published online: January 22, 2016

