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Insight into the 6-Thiopurine-Mediated Termination of the Invasive Motility of Tumor Cells Derived from Inflammatory Breast Cancer

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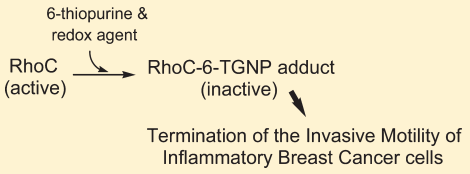
ABSTRACT: Our study showed that a combination of 6-thiopurine (6-TP) drugs and a redox agent effectively inhibits the motility of SUM cells derived from human inflammatory breast cancer (IBC) cells and RhoC-overexpressed mammary epithelium cells. This 6-TP-mediated inhibition of cell motility occurs because the treated 6-TPs target and inactivate RhoC. A molecular mechanism for inactivation by the 6-TP-mediated RhoC is proposed by which treated TPs are converted in cells into 6-thioguanosine phosphate (6-TGNP). This 6-TGNP in turn reacts with the Cys^{20} side chain of the redox-sensitive GXXXCGK(S/T)C motif of RhoC to produce a 6-TGNP-RhoC disulfide adduct. A redox agent synergistically enhances the formation process of this disulfide. The adduct that is formed impedes RhoC guanine nucleotide exchange, which populates an inactive RhoC. Our results suggest that 6-TGNP can also react with the redox-sensitive GXXXCGK(S/T)C and GXXXXGK(S/T)C motif of RhoA and Rac, respectively, to produce a 6-TGNP-RhoA and 6-TGNP-Rac disulfide adduct. However, given that RhoC has been shown to be overexpressed in ~90% of IBC lesions, the populated RhoC but not other Rho proteins is likely to be a primary target for 6-TPs and a redox agent to terminate the metastasis of IBC.



RhoC, a member of the Rho family of GTPases, has been shown to be overexpressed in ~90% of human inflammatory breast cancer (IBC) lesions.[1,2] RhoC has increasingly attracted clinical interest because of the emerging evidence of its metastatic role in IBC, which is the most lethal form of locally advanced breast cancer and annually accounts for ~6% of the new breast cancer cases in the United States.[3] RhoC also plays a role in the metastasis of various other tumors, including hepatocellular and colon carcinomas.[4,5] Misregulation of other Rho GTPases, such as RhoA and Rac, also is linked to many disorders, including cancers and heart and lung diseases.[6−14]

Like the Ras family of GTPases, Rho GTPases function by cycling between inactive GDP-bound and active GTP-bound states.[15] Various protein regulators such as guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins control this GDP/GTP cycling.[16] GEFs enhance the guanine nucleotide exchange (GNE) of these GTPases.[16] Dbl's big sister (Dbs), one of the Rho-specific GEFs, has been shown to be a RhoC GEF.[17] Because cells contain relatively higher concentrations of GTP than of GDP,[18] the GEF-mediated GNE of these GTPases populates the GTP-loaded active GTPases in cells. GTPase-activating proteins stimulate hydrolysis of the γ-phosphate of the bound GTP to produce inactive GDP-bound GTPases and free phosphates.[16]

When small GTPases are redox sensitive, a cellular redox agent functions as their regulator.[15] Most Rho proteins, including Rac1 and Cdc42, are redox sensitive because they possess a single redox-sensitive cysteine (Cys^{18}, Rac1 numbering) in the GXXXXGK(S/T)C motif (monothiol).[19] A subset of the GXXXXGK(S/T)C motif is found in RhoA and RhoB.[20] This subset contains a secondary cysteine (Cys^{16}, RhoA numbering) in addition to the primary redox-sensitive cysteine (Cys^{20}, RhoA numbering, which is equivalent to the Rac1 Cys^{18}) termed the GXXXCGK(S/T)C motif (dithiol). Although both the GXXXXGK(S/T)C and GXXXCGK(S/T)C motifs have the same redox sensitivity,[19] the latter has an additional redox modulation function.[20] An analysis of the RhoC crystal structure PDB 2GCO[21] in conjunction with a sequence analysis indicates that RhoC also possesses the GXXXCGK(S/T)C motif. Ras GTPases contain a distinct redox-sensitive NKCD motif.[22] Intriguingly, the RhoC Cys^{20} (the RhoC numbering is the same as the RhoA numbering) site is located at the Rho nucleotide-binding site,[21] but Ras Cys^{118} (Harvey Ras numbering) is remote from the Ras nucleotide-binding site.[23] Redox agents include nitric oxide (NO), nitrogen dioxide ($^{\bullet}NO_2$), and dinitrogen trioxide (N_2O_3) as well as superoxide anion radical ($O_2^{\bullet-}$) and hydrogen peroxide.[15] Of these redox agents, $^{\bullet}NO_2$ and $O_2^{\bullet-}$ have been shown to directly target these redox sensitive residues of Rho and Ras proteins.[15]

6-Thiopurine (6-TP) prodrugs, including 6-thioguanine (6-TG) and azathioprine, are antimetabolites. They are widely used to treat acute lymphoblastic leukemia, and autoimmune disorders such as inflammatory bowel disease.[24−26] In cells, cellular enzymes[24−29] convert inactive prodrug 6-TPs into the pharmacologically active 6-thioguanine nucleotide that can be grouped into deoxy-6-thioguanosine phosphate (6-TdGNP) and 6-thioguanosine phosphate (6-TGNP). Furthermore, depending on the number of ribose phosphates, 6-TGNP can be further classified as

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6-thioguanosine diphosphate (6-TGDP) and triphosphate (6-TGTP). 6-TdGNP can be incorporated into the *de novo* synthesis of DNA as a form of 6-TG. 6-TG in DNA can then be recognized as a DNA lesion by the mismatch repair system, a recognition that results in induction of the mismatch repair-mediated cell apoptosis.[30−32] This 6-TG-mediated induction of mismatch repair is believed to be the main mechanism for the action of 6-TPs in the treatment of acute lymphoblastic leukemia. In contrast to 6-TdGNP, neither the metabolic path of 6-TGNP nor its therapeutic activity and/or cytotoxicity has been clearly established. A few recent studies have addressed the action of 6-TGNP on small GTPases. We have recently shown that long-term treatment of Ras-activated tumor cells, such as bladder carcinoma (cell-line, T24) and fibrosarcoma (cell-line, HT1080), with 6-TG results in production of cellular 6-TGNP that targets Ras GTPase.[33] This Ras-targeting action of 6-TGNP results in downregulation of Ras, which in turn terminates the tumorous growth of these cells. This Ras-targeting action of 6-TGNP extends beyond its effect on tumor cells and is considered cytotoxic[33] because it deregulates the Ras GTP/GDP cycle. It also has been shown that 6-TGNP targets and inactivates Rho GTPases such as Rac1.[28,34] This Rho GTPase-targeting action of 6-TGNP may be attributable to the therapeutic effect of 6-TPs on the immune system as well as on inflammatory bowel disease.[35−37] The study shows that the therapeutic action of 6-TP in inflammatory bowel disease is correlated with the Rac1-targeting 6-TGNP that blocks Rac1 GNE by its GEF Vav;[34] however, the details of the molecular mechanism by which this occurs is unknown.

In this study, we have examined the potential therapeutic action of 6-TGNP, derived from a 6-TP prodrug such as 6-TG, on IBC-derived tumor cells via targeting the redox-sensitive GXXXCGK(S/T)C motif of RhoC. We have also examined the synergistic role of a redox agent on the action of 6-TGNP on RhoC. The results lead to the opening of a new paradigm for a therapeutic application of 6-TGNP with a redox agent for treatment of IBC caused by an overexpression of RhoC. On the basis of our results, we propose a mechanism that explains the 6-TGNP-mediated inactivation of RhoC. This proposed mechanism also explains the previously observed inhibitory action of 6-TP drugs on Rac1 via blockage of the Vav-mediated Rac1 GNE.[28,34]

MATERIALS AND METHODS

Cell Culture and Treatments. SUM cells were cultured according to the protocol provided by the vendor (Asterand, Detroit, MI). Transfection of human mammary epithelial cells with wild type RhoC and mutant C20S RhoC to produce HME-RhoC and HME-C20S RhoC, respectively—as well as the culturing of these HME-RhoC and HME-C20S RhoC cells—was performed according to procedures explained in the previous study.[38] Human colon adenocarcinoma (cell line, SW480) and hepatocellular carcinoma (cell line, HCCLM3) cells were cultured according to the protocol provided by the vendor (American Type Culture Collection, Manassas, VA). Cells were treated with 6-TG (1 μM) and/or a NO-releasing agent diethylenetriamine/nitric oxide (DETA/NO) (10 μM) or *S*-nitrosoglutathione (GSNO) (10 μM) at every ∼24 and/or ∼20 or ∼8 h, respectively, for 3 or 4 days to maintain a minimal 50% of the initial concentration of NO in the culture media.[33] The treatment time interval for 6-TG is based on empirical reasoning that three consecutive treatments with 6-TG for up to 4 days will not result in an overdose of 6-TG but will ensure that the level of 6-TG is higher than 1 μM in the culture media. The established cell treatment time interval for DETA/NO or GSNO is because the half-life of DETA/NO and GSNO is ∼20 and ∼8 h, respectively, at pH 7.4 and 37 °C.[39−41]

Cell Motility Assay. An invasion assay using Matrigel (BD Biosciences, Bedford, MA), was performed, with minor modifications, as previously described[42] and was the major method used in examining the effect of 6-TG and/or a redox agent on SUM149, HME-RhoC, and HME-C20S RhoC cells. The top chamber of a Transwell filter (6.5 mm with 8 μm pores, Costar; Corning, NY) was coated with a 10 μL aliquot of 10 mg/mL Matrigel, and the lower chamber of the Transwell was filled with either serum-free or serum-containing media. Sample cells were prepared so as to resuspend cells in a serum-free medium with 0.1% BSA at a concentration of ∼4×10^5 cells/mL. The sample cells (0.2 mL) were added to the top chamber of a Transwell filter. The cells in the top chamber were treated with a reagent solution (0.1 mL) containing 6-TG and/or DETA/NO or GSNO in serum-free media and then incubated for 3 days at 37 °C in a 10% CO_2 incubator. When necessary, the top chamber was repeatedly treated with the reagent solution (0.1 mL) as indicated above. To ensure that the top chamber cells were evenly treated with the reagent(s), the cell treatment process included overlaying the reagent solution on the top chamber cells followed by a gentle mixing, by means of a pipettor, of the sample cells with the reagent solution. As a control, a serum-free media (0.1 mL), instead of the reagent solution, was also used to treat the top chamber cells repeatedly in the same manner as the reagent solution was used. To complete the examination of cell motility in the presence and absence of 6-TG and/or DETA/NO or GSNO for 3 days, transwell filters were fixed with methanol and stained with hematoxylin and eosin, and cells in the serum-containing samples were counted. The number of cells that had invaded the serum-free, medium-containing lower chambers was used as a background.

The Colorimetric-based QCM Cell Invasion Assay Kit (Millipore, Billerica, MA) was used for the time-dependent cell migration assay of SUM149, SW480, and HCCLM3 cells. Serum-free sample cells (∼2×10^5 cells/mL) were prepared as indicated in the section of the Matrigel invasion assay. The serum-free sample cells (0.25 mL) were loaded into the rehydrated upper chamber. The cell-containing upper chamber was then overlaid with a solution (0.05 mL) containing 6-TG and/or DETA/NO or GSNO. Serum-free or serum-containing media (0.5 mL) were added to the lower chamber. The sealed plate was then incubated for one to 4 days at 37 °C in a 10% CO_2 incubator. As with the Matrigel invasion assay, the upper chamber was repeatedly overlaid with the solution (0.05 mL) containing 6-TG and/or DETA/NO to maintain the desired level of 6-TG and NO. The fraction of cells that migrated from the upper to the lower chamber was then measured colorimetrically according to the procedure provided by the manufacturer.

Viability and Apoptotic Assays. A cell viability assay employing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)[43,44] and an apoptosis assay using the ApoAlert Caspase-3/8 Colorimetric Assay Kit (Takara, Japan) were performed for SUM149, HME-RhoC, and HME-C20S RhoC cells. These cells (∼1×10^5 cells/mL) were treated with DETA/NO and/or 6-TG for 3 days. An apoptosis inducer, 2-amino-*N*-quinolin-8-yl-benzenesulfonamide (10 μM), was used for positive control of the caspase assay.

RhoC Activity Assay in Cells. Cells were lysed in an extraction buffer containing 50 mM NaCl, 5 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM diethylenetriaminepentaacetic acid (DTPA), and 0.1% NP-40 in 150 mM TrisHCl (pH 8.0). The content of active RhoC present in the cells was determined by Western blot analysis using a monoclonal anti-RhoC antibody (Biocompare, South San Francisco, CA). To determine RhoC activity, the colorimetric RhoA activation assay Biochem Kit (Cytoskeleton, Denver, CO) was used essentially as described in the previous study,[45] except that the RhoC specific antibody (vide supra), instead of the RhoA antibody included in the assay kit, was used for this analysis.

Mass Spectrometric Analysis. Cell lysates in an extraction buffer were incubated with a monoclonal anti-RhoC antibody precoupled to glutathione−agarose beads. The beads were collected by centrifugation and washed three times with the extraction buffer. RhoC proteins on beads were then eluted with a solution containing 50% methanol, 0.1% formic acid, 0.1 mM $MgCl_2$ 1 mM EDTA, and 0.1 mM DTPA. The protein portion and RhoC-released nucleotide were separated by brief centrifugation (3000*g* for 5 min). The protein portion from the immunoprecipitated RhoC was resuspended in a buffer containing 1 mM EDTA and 0.1 mM DTPA in 50 mM TrisHCl (pH 7.4), then digested with trypsin for 10 h in the presence and absence of dithiothreitol (DTT, 10 mM), and analyzed with electrospray mass spectrometry (ESI-MS) and tandem mass spectrometry (MS/MS) in the positive ion mode ($[\text{molecular mass} + H]^+$).

Kinetic Assays. A transition metal-free assay buffer and vials as described in the previous study[33] were used for all kinetic assays. The kinetic assay buffer contained the highest grade of 50 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, and 0.1 mM DTPA in 50 mM TrisHCl (pH 7.4). Before any of the assays were performed, all protein samples were dialyzed with a metal-free buffer under anaerobic conditions. As with RhoA and Rac1,[19] RhoC proteins were expressed in *E. coli* and purified using anionic and size exclusion columns.

(a) A competitive binding assay was performed to determine the binding affinity of Rho GTPase and 6-TGDP. The radiolabeled [^{3}H]GDP-loaded Rho protein (1 μM) was titrated with various concentrations of 6-TGDP. The titrated Rho protein sample then was spotted onto a nitrocellulose membrane. Each membrane was washed three times with the assay buffer, and radioactivity was determined using a Beckman-Coulter scintillation counter. The radioactivity of the sample (Rho protein-bound [^{3}H]GDP) was converted into, and thus plotted, as the fraction of mol GDP per mol total Rho protein. The apparent dissociation constant ($^{app}K_D$) of the Rho protein with [^{3}H]GDP in the presence of 6-TGDP was determined by using Prism Software to fit the titration curve to a hyperbola.

(b) The effect of redox agents such as NO, $^{\bullet}NO_2$, and N_2O_3 on the binding interaction between RhoC proteins and GDP or 6-TGDP was examined under nitrogen gas-filled serum-stoppered anaerobic experimental conditions (O_2 < 3 ppm). These anaerobic experimental conditions were necessary to block the reaction of NO or $^{\bullet}NO_2$ with O_2 to produce higher oxides. The amount of NO and $^{\bullet}NO_2$ respectively in the sealed assay vials was determined by using a hemoglobin assay and NO_2^-/NO_3^- Assay Kit-C II (Dojindo) under anaerobic conditions.[19,46] N_2O_3 was generated by mixing NO and $^{\bullet}NO_2$ 1:1 stoichiometrically. However, because the N_2O_3 thus formed can be decomposed into NO and $^{\bullet}NO_2$, the notation of N_2O_3 in our experimental results does not necessarily indicate that the amount of N_2O_3 is 100%. To examine the redox property of RhoC with GDP, the [^{3}H]GDP-loaded RhoC protein (1 μM) was treated with redox agents (~3 μM) in the presence of free GDP (10 μM). The fraction of the redox agent-mediated release of the bound [^{3}H]GDP per Rho protein over time was determined as noted above, and the result was plotted against time. The rates of RhoC GDP dissociation in the presence of redox agents were determined by using Prism Software to fit the result to a one-phase exponential decay.

(c) To test the Rho 6-TGDP-binding interaction in the presence of redox agents, the 6-TGDP-loaded RhoC protein was treated with redox agents in the presence of free [^{3}H]GDP (10 μM). An association of RhoC protein with [^{3}H]GDP to produce a RhoC-[^{3}H]GDP complex will occur proportionally after RhoC releases 6-TGDP through the action of redox agents. Therefore, the fraction of the redox agent-mediated release of the bound 6-TGDP per Rho protein over time can be deduced by determination of the RhoC-bound [^{3}H]GDP, where the quantity of RhoC-bound 6-TGDP equals 1 minus the fraction of the RhoC-bound [^{3}H]GDP that has been determined as described in section b. The estimated data associated with the quantity of RhoC-bound 6-TGDP then was fitted to a one-phase exponential decay; Prism Software was used to derive the rate of 6-TGDP dissociation from RhoC.

(d) The effect of $^{\bullet}NO_2$ on the dissociation of 6-TGDP from RhoC was determined by treating the RhoC-6-TGDP complex titration with various concentrations of [^{3}H]GDP in the presence and absence of a reducing agent of either DTT or β-mercaptoethanol. The data were fitted to a hyperbola with Prism Software to estimate the $^{app}K_D$ of RhoC with 6-TGDP in the presence of [^{3}H]GDP.

RESULTS

Effects of Thiopurines on SUM and RhoC-Overexpressed HME Cells in the Presence and Absence of a Redox Agent. SUM149 cells and HME-RhoC have exhibited an invasive cell motility (Figure 1A). Unlike SUM149 and HME-RhoC cells, SUM102 and HME cells that are not overexpressed with RhoC do not exhibit cell motility. The observed RhoC-dependent cell motility of these SUM149 and HME-RhoC cells is consistent with the previous study.[38]

Unlike the action of 6-TG on fast-growing tumor cells,[33] the viability of SUM149 and HME-RhoC cells was minimally affected by treatment with 6-TG for 3 days (Figure 2A). The apoptotic effect of 6-TG on SUM149 and HME-RhoC cells also was minimal (Figure 2B). This result is similar to the effect of 6-TG on primary breast carcinoma in which treatment with 6-TG concentrations of less than ~2 μM for 3 days has minimal effect on the survival of primary breast carcinoma cells.[47] However, continuous treatment of SUM149 and HME-RhoC cells with 6-TG for 3 or more consecutive days resulted in a gradual but significant diminution of cell motility (Figure 1A). Despite the diminution of this activity, the level of total RhoC expression in these SUM149 and HME-RhoC cells was unchanged

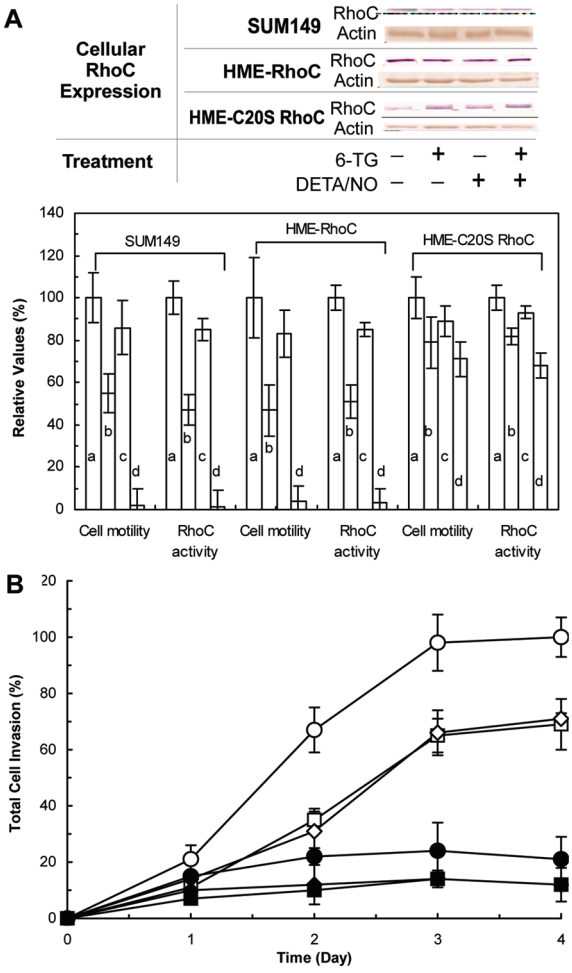

Figure 1. Determination of cell invasiveness in the presence and absence of 6-TG and/or DETA/NO. Experimental procedures for the determination of expression and activity of RhoC in cells treated with 6-TG (1 μM at every 24 h) and/or DETA/NO (10 μM at every 20 h) as well as the corresponding cell motility are described in the Materials and Methods section. (A) Matrigel invasion assay: the average number and standard error of triplicate measurements of SUM149, HME-RhoC, and HME-C20S RhoC cells that migrated through the Transwell filter pores to the lower chambers in the absence of 6-TG and DETA/NO was determined to be 176 $\pm$ 21, 160 $\pm$ 36, and 192 $\pm$ 19, respectively. For each cell line, invasive cell results were normalized against the average value—set at 100%—of the cell invasion that occurred in the absence of 6-TG and DETA/NO: a, cells untreated with 6-TG and DETA/NO; b, cells treated with only 6-TG; c, cells treated with only DETA/NO; and d, cells treated with both 6-TG and DETA/NO. Western blot analysis representing the total expression of RhoC in these cells also is shown. (B) QCM cell invasion assay: the maximal mean value determined for the optical density and standard error of triplicate measurements corresponding to the migration of SUM149 cells from the upper to the lower chamber through filter pores without treatment with 6-TG and DETA/NO on day four of experiment was estimated to be 1.13 $\pm$ 0.16. All colorimetrically determined optical density values associated with cell migration under these experimental conditions were normalized against the mean value of optical density for the value of the SUM149 cell migration in the absence of 6-TG and DETA/NO that was set at 100%: SUM149 cells untreated (○) or treated (●) with 6-TG and DETA/NO; SW480 cells untreated (□) or treated (■) with 6-TG and DETA/NO; and HCCLM3 cells untreated (◇) or treated (◆) with 6-TG and DETA/NO. For both (A) and (B), all data represent average and standard error values from triplicate measurements.

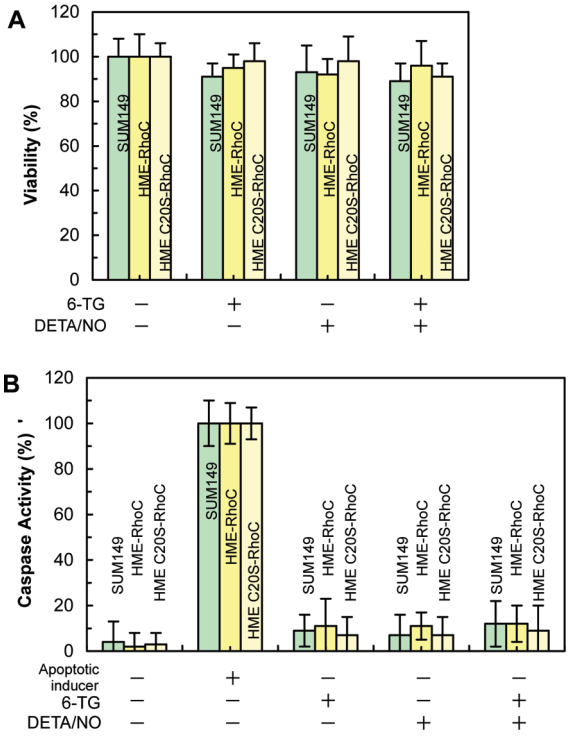

Figure 2. Effect of 6-TG in combination with DETA/NO on the viability and caspase activity of cells. MTT and caspase assays for cells treated with 6-TG (1 μM at every 24 h) and/or DETA/NO (10 μM at every 20 h) were performed as described in Materials and Methods. (A) MTT assay: for each cell line, the determined values of cell viability in the presence of 6-TG and/or DETA/NO were normalized against the mean value of cell viability in the absence of 6-TG and DETA/NO (set to be 100%). (B) Caspase-3/8 assay: the apoptotic activity of sample cells treated with the apoptotic inducer was set to be 100%, and other results were then expressed as normalized values against the mean value associated with the treatment with the apoptotic inducer. For both (A) and (B), all values represent the mean values and standard error values from triplicate measurements.

(Figure 1A). Because cell motility is coupled with the activity of RhoC, but not the level of RhoA expression, this diminution of cell motility is likely correlated with the 6-TG-mediated deregulation of RhoC activity.

Invasive cell motility also can be achieved by an overexpression of the mutant RhoC C20S in HME (i.e., HME-C20S RhoC) cells (Figure 1A). However, the effect of 6-TG on the motility of HME-C20S RhoC cells was insignificant compared with the effect of 6-TG on the motility of SUM149 and HME-RhoC cells (Figure 1A). The activity of, and total expression of, RhoC C20S also were unchanged by the treatment of HME-C20S RhoC with 6-TG for 3 days (Figure 1A). This result suggests the possibility that the RhoC residue Cys^{20} somehow plays a role in the action of the 6-TG-mediated diminution of the motility of SUM149 and HME-RhoC cells.

The cell viability and caspase activity of these SUM149, HME-RhoC, and HME-C20S RhoC cells were minimally changed by treatment with the NO donor alone (Figure 2). A minor effect of the NO donor DETA/NO on the viability of head and neck squamous cell carcinoma (cell-line HNSCC) was also observed;[48] however, this effect may depend on the type of cell involved. The effect of 3 days of continuous treatment with the NO donor on the invasive cell motility and RhoC activity of these

cells also was minimal (Figure 1A). Note that both SUM149 and HME-RhoC cells treated with a NO donor DETA/NO or GSNO initially had increased motility (10−30%) within 5 h; however, after 10 h, this enhanced motility declined slightly below the original level. This initial activation may be a result of the redox response of RhoC as this protein possesses the GXXXCGK(S/T)C motif.[20] This notion is supported because the cellular activity of C20S RhoC was unchanged after treatment of HME-C20S RhoC cells with a NO donor; the initial stimulatory effect of a NO donor on HME-C20S RhoC cells was not observed.

Complete termination of the invasive cell motility of SUM149 and HME-RhoC cells was observed when these cells were treated for 3 days with both 6-TG and DETA/NO (or GSNO) (Figure 1A). The viability and caspase activity of SUM149 and HME-RhoC cells were minimally affected by treatment with 6-TG and DETA/NO (Figure 2). This means that the 6-TG-mediated termination of the invasive motility of these cells in the presence of the NO donor cannot be attributed to a change in the viability and/or induction of apoptosis of these cells by 6-TG and DETA/NO. Just as with the single treatment with 6-TG or a NO donor, the combined effect of 6-TG and a NO donor is likely linked to the redox-sensitive GXXXCGK(S/T)C motif of RhoC. This speculation reflects the comparative lack of a significant effect of 6-TG with a NO donor on either the mutant C20S RhoC activity or the invasive cell motility of HME-C20S RhoC cells compared with the effect of either 6-TG or a NO donor alone on the C20S RhoC or on these same HME-C20S RhoC cells (Figure 1A).

As with SUM149 cells, most of the invasive motility of SW480 and HCCLM3 tumor cells associated with RhoC[4,49] was terminated by treatment with 6-TG in combination with a NO donor for 4 days (Figure 1B). As with SUM149 cells (Figure 1A), the level of expression of RhoC in these cells was unchanged, but the activity of RhoC was drastically diminished when these cells were treated with 6-TG and a NO donor (not shown). This result suggests that, regardless of tumor types, invasive cell motility mediated by RhoC can universally be terminated by the action of 6-TG with a NO donor.

Analysis of RhoC−6-TGNP Adduct from Cells Treated with 6-TG and/or a Redox Agent. To explore the underlying mechanism of the 6-TG-mediated inhibition of the cell motility of SUM149 and HME-RhoC in the presence of a redox agent, RhoC protein was isolated from these cells treated with or without 6-TG in the presence or absence of a redox agent; this protein then was digested, and its peptides were analyzed with ESI-MS. A mass peak 1481.6 Da, assigned to be a RhoC-derived peptide−6-TGDP disulfide adduct (TC^{20}LLIVFSK−6-TGDP) (Figure 3B), was commonly exhibited in co-immunoprecipitated (co-IPed) RhoC samples isolated from both SUM149 cells (Figure 3A, upper panel of the left column) and HME-RhoC cells treated with 6-TG (Figure 3A, upper panel of the right column). In the presence of a NO donor, the peak assigned to be a RhoC−6-TGDP adduct also was increased, suggesting that a NO donor enhances the formation of the RhoC−6-TGDP adduct. However, this 1481.6 Da mass peak was not found in control cell samples that were untreated with 6-TG (Figure 3A, lower panel of the left and right columns). This result suggests that in SUM149 and HME-RhoC cells, 6-TGDP, which is derived from treated 6-TG, targets the Cys^{20} side chain of the redox sensitive GXXXCGK(S/T)C motif of RhoC to produce a RhoC−6-TGDP adduct.

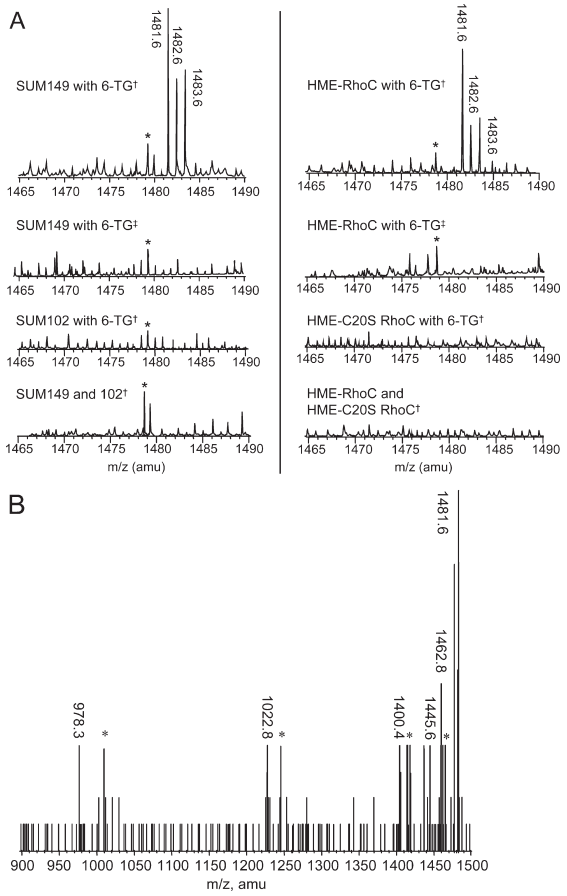

Figure 3. Detection of unusual 6-TGNP adduct from SUM and HME-RhoC cells. The ESI-MS and MS/MS methods to detect and identify a chemically modified RhoC residue are described in the Materials and Methods section. (A) A mass peak of 1481.6 Da was only detected when the DTT-free trypsin-digested ESI-MS sample was prepared with co-IP using the RhoC antibody from the 6-TG-treated SUM149 cells or from the 6-TG-treated HME-RhoC cells in the absence of DTT. After consideration of the mass of the peak in conjunction with the sample preparation conditions, the best candidate to account for the spectrum of the mass peak at 1481.6 Da is a RhoC fragment TC^{20}LLIVFSK cross-linked with 6-TGDP (TC^{20}LLIVFSK−6-TGDP disulfide adduct). †Trypsin digestion in the absence of DTT. ‡Trypsin digestion in the presence of DTT. *Unassigned mass peaks. (B) An MS/MS analysis was performed with the DTT-free trypsin-digested ESI-MS sample of 6-TG-treated SUM149 cells to identify a biomolecule that has a mass of 1481.6 Da. Major MS/MS peaks shown were best fitted to the masses of ion fragments of the TC^{20}LLIVFSK−6-TGDP adduct: (i) the 1462.8 Da fragment, formed upon loss of H_2O from the β-phosphate of the 6-TGDP moiety of TC^{20}LLIVFSK−6-TGDP adduct; (ii) the 1445.6 Da fragment, formed by losing an H_2O and an OH from the β- and α-phosphate of the 6-TGDP moiety of TC^{20}LLIVFSK−6-TGDP adduct; (iii) the 1400.4 Da fragment, formed upon loss of COOH from the C-terminus of the TC^{20}LLIVFSK−6-TGDP adduct as well as an H_2O and an OH from the β- and α-phosphate of the 6-TGDP moiety of TC^{20}LLIVFSK−6-TGDP adduct; (iv) the 1022.8 Da fragment, formed upon loss of the 6-TGDP moiety from the TC^{20}LLIVFSK−6-TGDP adduct; and (v) the 978.3 Da fragment, formed by losing the peptide C-terminus COOH and the 6-TGDP moiety from the TC^{20}LLIVFSK−6-TGDP adduct. Taking into consideration the MS/MS result under our experimental conditions (e.g., SUM149 cells treated with 6-TG), the 1481.6 Da molecule detected in the ESI-MS analysis is assigned to the TC^{20}LLIVFSK−6-TGDP disulfide adduct. *Unassigned mass peaks.

In contrast to SUM149 cells, the RhoC mRNA expression in SUM102 cells has been shown to be low.[1] However, RhoC, which is minimally expressed in SUM102 cells, also could react with 6-TGDP to produce the 1481.6 Da peak (Figure 3A, middle panel of the left column). Moreover, other Rho GTPases, such as RhoA and RhoB, possess the redox-sensitive GXXXCGK(S/T)C motif with an identical sequence of TC^{20}LLIVFSK. Hence, it also is possible that the origin of the 1481.6 Da peak from the SUM102 sample can be derived from the reaction between 6-TGDP and the endogenously present RhoA and/or RhoB. Because C20S RhoC lacks the redox-sensitive Cys^{20} residue, detection of the RhoC−peptide adduct in HME-C20S RhoC cells was not expected. However, a low intensity but definite 1481.6 Da peak was also observed when HME-C20S RhoC cells were treated with 6-TG (Figure 3A, middle panel of the right column). As with SUM102 cells, the endogenously expressed RhoA and RhoB in HME-C20S RhoC cells may be a target of 6-TGDP and subsequently produce a RhoA− and/or RhoB−6-TGDP adduct.

Because cells maintain a ratio of GTP/GDP larger than 1,[18] the cellular concentration of 6-TGTP also is likely to exceed that of the 6-TGDP. However, ESI-MS analysis has detected a RhoC fragment TC^{20}LLIVFSK with 6-TGDP (TC^{20}LLIVFSK−6-TGDP) but not with 6-TGTP (TC^{20}LLIVFSK−6-TGTP) (Figure 3A). This is likely because the γ-phosphate of 6-TGTP is so unstable that preparation of the ESI-MSI sample, particularly the digestion at room temperature of the co-IPed RhoC fraction with trypsin, results in conversion of the 6-TGTP into 6-TGDP that covalently attaches to the RhoC Cys^{20} side chain.

Kinetic Properties of Rho GTPases with 6-TGNP in the Presence or Absence of a Redox Agent. To better understand the 6-TGNP-mediated inactivation mechanism of RhoC with or without a redox agent, a redox-based biochemical analysis was performed for Rho GTPases, including RhoC and RhoA, with 6-TGDP.

A competitive binding study shows that GDP bound to RhoA and RhoC can be displaced with 6-TGDP (Figure 4A), similar to what happens with Ras GDP with 6-TGDP.[33] The RhoC- and mutant RhoC-bound GDP also can be competitively displaced with 6-TGDP (Figure 4A). The binding affinities that 6-TGDP has for all of these examined Rho proteins are ~2-fold weaker than that of GDP for Rho proteins (Figure 4A). However, the range of the values determined for the true dissociation constant ($^{true}K_D$) of Rho proteins for 6-TGDP, including C20S RhoC (Figure 4A), nevertheless indicates that 6-TGDP has a high affinity for binding to Rho GTPases.

None of the redox agents tested were able to enhance GDP dissociation from C20S RhoC (Figure 4B). Rates of the NO- or N_2O_3-mediated GDP dissociation from RhoC were minimal (Figure 4B). However, $^{\bullet}NO_2$ enhances dissociation of GDP from RhoC (Figure 4B). These results were consistent with a previous study suggesting that $^{\bullet}NO_2$, but neither NO nor N_2O_3, targets the GXXXXGK(S/T)C and GXXXCGK(S/T)C motif of the redox-sensitive Rho GTPases to enhance Rho GDP dissociation.[19,20] $^{\bullet}NO_2$, but neither NO nor N_2O_3, enhances dissociation of 6-TGDP from the redox inert C20S RhoC (Figure 4C). Our previous study[19] provides an explanation for this result in which $^{\bullet}NO_2$ targets the sulfur atom of the bound 6-TGDP, rather than the redox inert C20S RhoC protein, to produce the 6-TGDP−NO_2 adduct that can be degraded into 5-guanidino-4-nitroimidazole diphosphate. Dissociation of 6-TGDP from RhoC and mutant RhoC C20S by NO or N_2O_3 was minimal (Figure 4C).

Because of the result associated with the action of $^{\bullet}NO_2$ on the GDP-bound RhoC combined with the 6-TGDP-bound C20S RhoC, the greater effectiveness of the target action of $^{\bullet}NO_2$ on a RhoC−6-TGDP complex is predictable. This predictability is because both the ligand and receptor of the RhoC−6-TGDP complex, 6-TGDP and RhoC, respectively, are redox sensitive. Unexpectedly, however, a much slower rate of $^{\bullet}NO_2$-mediated 6-TGDP dissociation from RhoC was observed compared with that of 6-TGDP dissociation from C20S RhoC or GDP dissociation from RhoC (Figure 4C). To better understand this enigmatic result, the 6-TGDP-loaded RhoC was pretreated with $^{\bullet}NO_2$, and a competitive displacement of the preloaded 6-TGDP with GDP was performed. Only a minimal fraction of the preloaded 6-TGDP was dislodged with GDP (Figure 4D). Dbs, the RhoC GEF, also was unable to displace the bound 6-TGDP with GDP. This blockage of dissociation of the bound 6-TGDP from RhoC was not observed when the RhoC−6-TGDP complex was untreated or pretreated with NO or N_2O_3. The mass peak 1481.6 Da identified in cell samples treated with 6-TG (Figure 3A) also was found in this *in vitro* kinetic study sample of RhoC−6-TGDP that was treated with $^{\bullet}NO_2$ and trypsin but not with either NO or N_2O_3. The 6-TGDP in the $^{\bullet}NO_2$-treated 6-TGDP C20S RhoC complex can be displaced with GDP (Figure 4D). The kinetic results with RhoC and mutant RhoC in conjunction with MS data suggest that the $^{\bullet}NO_2$-mediated formation of the RhoC Cys^{20}-6-TGDP disulfide adduct is linked to blockage of the dissociation of the 6-TGDP from RhoC in the presence and absence of Dbs.

A treatment of the $^{\bullet}NO_2$ pretreated-RhoC−6-TGDP complex with reducing agent DTT (or β-mercaptoethanol) results in the removal of 6-TGDP from the RhoC nucleotide-binding site and replacement with GDP (Figure 4D). This is likely because DTT or β-mercaptoethanol reduces and thus disrupts the disulfide bond between 6-TGDP and the RhoC Cys^{20} side chain. This unlinked 6-TGDP can then be liberated from the RhoC protein, a result that is consistent with the disulfide blockage hypothesis.

Potential Mechanism for Formation of the RhoC−6-TGDP Adduct. It is possible to hypothesize that the formation of the RhoC Cys^{20}−6-TGDP disulfide adduct traps 6-TGDP at the RhoC nucleotide-binding site, resulting in the failure of 6-TGDP dissociation from RhoC. This hypothesis is tenable because the C_6 oxygen atom of the bound GDP (C_6 sulfur atom in case of 6-TGDP) is vicinal to the sulfur atom of the RhoC Cys^{20} side chain that is located at the RhoC nucleotide-binding site.[21] The RhoC crystal structure (PDB 2GCO)[21] suggests that only one nucleotide (e.g., GDP or 6-TGDP) can be bound to RhoC, and thus only one RhoC Cys^{20}−6-TGDP adduct can be formed. This hypothesis on the formation of the RhoC Cys^{20}−6-TGDP disulfide adduct explains the enigmatic kinetic results shown in Figure 4C. Unlike with RhoC C20S, a fraction of the Cys^{20} side chain of RhoC reacts with the bound 6-TGDP in the presence of $^{\bullet}NO_2$ to produce the RhoC Cys^{20}−6-TGDP disulfide adduct that blocks release of 6-TGDP from RhoC. This clarifies why 6-TGDP has a slower apparent $^{\bullet}NO_2$-mediated dissociation rate from RhoC than 6-TGDP has from RhoC C20S (Figure 4C). Yet, some other fractions of the 6-TGDP that are bound to RhoC could be dissociated from RhoC, and this dissociation then could be followed by the radical-based mechanistic process suggested for Rho proteins.[19] This notation is supported by the observation that the apparent dissociation rate of 6-TGDP from RhoC in the presence of $^{\bullet}NO_2$ is faster than the dissociation rate of 6-TGDP in the presence of NO or N_2O_3 (Figure 4C).

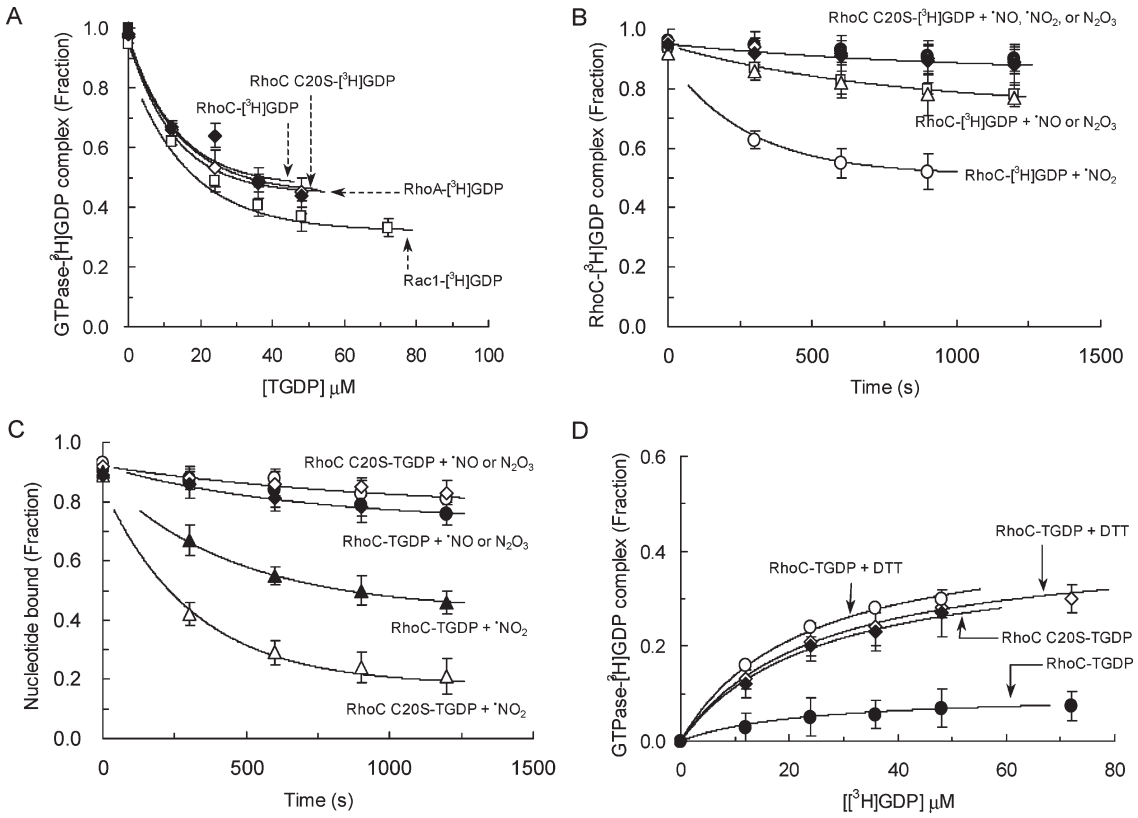

Figure 4. Kinetic properties of RhoC with 6-TGDP in the presence or absence of a redox agent. All kinetic analyses are described in the Materials and Methods section. (A) $^{app}K_D$ values of Rac1, RhoA, RhoC, and RhoC C20S for 6-TGDP were estimated to be 7.9, 11.6, 11.8, and 11.2 μM, respectively. $^{true}K_D$ values of Rac1, RhoA, RhoC, and RhoC C20S for 6-TGDP were then calculated to be 5.1, 10.8, 11.1, and 10.5 μM, respectively, by using a compensation equation, $^{true}K_D$ for 6-TGDP = $^{app}K_D$ for 6-TGDP/(1 + [[^{3}H]GDP]/$^{true}K_D$ for GDP),[33] in conjunction with the values given. (The $^{true}K_D$ values of Rac1 and RhoA for GDP were known to be 1.8 and 13.0 μM.[65] Within this study, the $^{true}K_D$ value of RhoC and RhoC C20S for GDP was determined be 15.0 ± 0.8 and 14.2 ± 0.8 μM, respectively.) (B) The rates of GDP dissociation from RhoC by NO, $^{\bullet}NO_2$, and N_2O_3 (a mixture of NO and $^{\bullet}NO_2$) were determined to be 0.19 × 10^{-3}, 6.04 × 10^{-3}, and 0.21 × 10^{-3} s^{-1}, respectively. The GDP dissociation rates from RhoC C20S by NO, $^{\bullet}NO_2$, and N_2O_3 were estimated to be 0.02 × 10^{-3}, 0.05 × 10^{-3}, and 0.04 × 10^{-3} s^{-1}, respectively. (C) The NO-, $^{\bullet}NO_2$-, and N_2O_3-mediated rates of 6-TGDP dissociation from RhoC by NO, $^{\bullet}NO_2$, and N_2O_3 were determined to be 0.08 × 10^{-3}, 2.65 × 10^{-3}, and 0.16 × 10^{-3} s^{-1}, respectively. The rates of 6-TGDP dissociation from RhoC C20S in the presence of NO, $^{\bullet}NO_2$, and N_2O_3 were estimated to be 0.04 × 10^{-3}, 6.27 × 10^{-3}, and 0.12 × 10^{-3} s^{-1}, respectively. (D) $^{app}K_D$ values of RhoC for [^{3}H]GDP in the presence or absence of DTT were estimated to be 22.4 and 103.7 μM, respectively. $^{app}K_D$ values of C20S RhoC for [^{3}H]GDP in the presence or absence of DTT were determined to be 24.8 and 25.2 μM, respectively. The regression values associated with these fits were >0.8595. All data points associated with vertical standard error bars shown in figures are the mean values of triplicate measurements.

The results of our *in vitro* kinetic study also show that $^{\bullet}NO_2$ only enhances formation of the RhoC Cys^{20}−6-TGDP disulfide adduct. This is consistent with previous studies showing that $^{\bullet}NO_2$ reacts with a thiolate to produce a thiyl radical.[50,51] The thiyl radical formed also can react with a thiolate to produce a disulfide radical anion.[52] Because a disulfide radical anion is a strong reductant, it predominantly reacts with O_2 to produce $O_2^{\bullet-}$ and a disulfide. On the basis of these previous studies, we propose a molecular mechanism for the formation of the RhoC−6-TGDP disulfide adduct in the presence of $^{\bullet}NO_2$ (Figure 5A). $^{\bullet}NO_2$ reacts with the RhoC Cys^{20} side chain to produce a RhoC Cys^{20} side chain thiyl radical. This RhoC radical then reacts with the sulfur of the RhoC-bound 6-TGDP in a 6-thioxo form to yield a RhoC Cys^{20} disulfide radical anion. This protein radical anion can be quenched by O_2 to produce a superoxide anion radical and the RhoC−6-TGDP disulfide adduct. It is also possible that the thiyl radical formed can further react with another thiyl radical to produce a disulfide molecule. Therefore, alternatively, if the 6-TGDP that possesses thiolate (a 6-sulfido form) is dominant, the sulfur atom of the bound 6-TGDP will be targeted by $^{\bullet}NO_2$ to produce a 6-TGDP thiyl radical. This 6-TGDP radical then reacts with the RhoC Cys^{20} side chain thiyl radical to produce the RhoC−6-TGDP disulfide adduct. However, because of the insignificant deviation of the $^{true}K_D$ value of RhoC for 6-TGDP from the value of RhoC for GDP, the state of the bound 6-TGDP is likely to be in a 6-thioxo form. Thus, formation of RhoC−6-TGDP disulfide via a direct targeting of the bound 6-TGDP is unlikely.

A nonradical-based process (Figure 5B) also is possible. However, because we were unable to detect formation of a disulfide bond between RhoC and the bound 6-TGDP in the presence of a transition metal (i.e., Fe^{2+} or Cu^{2+}) but absence of $^{\bullet}NO_2$ under *in vitro* experimental conditions, this nonradical-based mechanism is unlikely to occur.

All forms of 6-TGNP, such as 6-TGDP and 6-TGTP, have the same 6-TG moiety; they differ from each other only in the number of phosphates linked to the thionucleotide ribose. The proposed mechanism suggests that the results of kinetic

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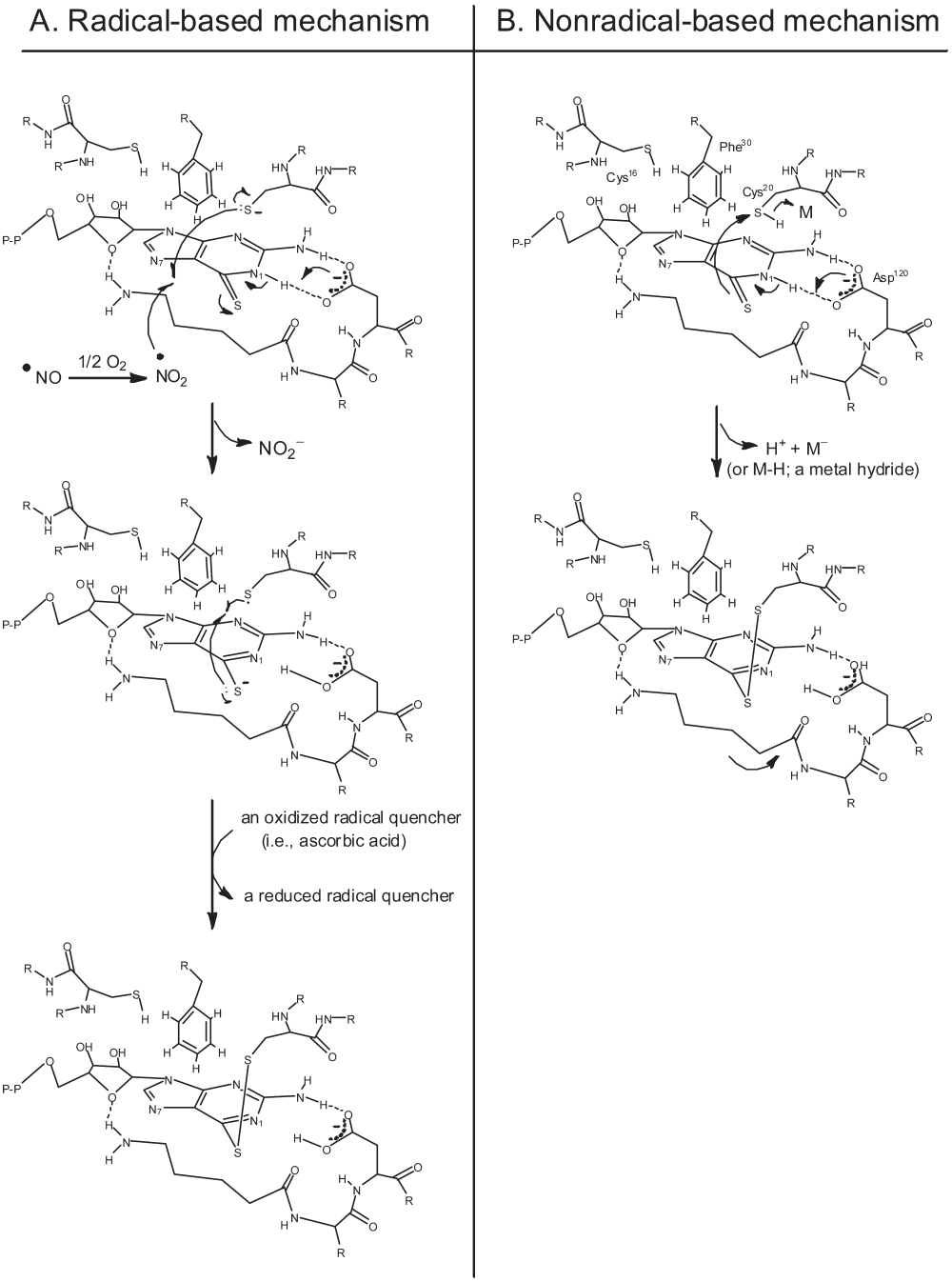

Figure 5. Proposed mechanisms of the Rho−6-TGNP disulfide adduction formation. A symbol M represents a transition metal, serving for an electron acceptor. The dotted lines represent putative hydrogen-bond interactions between RhoC residues and 6-TGNP.

analysis of 6-TGDP with RhoC shown in this study are intrinsic to the chemical properties of the thiolate moiety of 6-TG of 6-TGDP coupled with the RhoC Cys^{20} side chain. Hence, the biochemical features of other 6-TGNPs, such as 6-TGTP, with RhoC will not differ significantly from those of 6-TGDP with RhoC (Figure 4).

DISCUSSION

RhoC is highly overexpressed in IBC, which is metastatic and characterized by extremely low rates of survival. Hence, early diagnosis and blockage of metastasis are the keys to increases in the rate of survival of IBC patients. A desirable chemotherapeutic agent for the RhoC-overexpressed IBC would target RhoC directly and inhibit it. Direct targeting of tumorigenic RhoC by such an agent could reduce cytotoxicity while maximizing the antitumor effect. However, no agent that targets RhoC directly is currently available.

This study shows that treatment with 6-TPs and a redox agent ceases the RhoC-mediated motility of cells, including SUM149 and HME-RhoC, that mimic the invasive metastasis of IBC cells. An explanation is also proposed for the underlying mechanism used by 6-TPs and a redox agent to target and inactivate RhoC in SUM149 and HME-RhoC cells.

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Mechanism of the 6-TG-Mediated Cessation of RhoC-Mediated Cell Motility. Our analysis suggests that the formation of a disulfide adduct between the bound 6-TGNP and the GXXXCGK(S/T)C motif of RhoC accounts for the RhoC-targeting anticell motility action of 6-TPs and a redox agent. In the presence of a redox agent, the 6-thioxo moiety of 6-TGNP cross-reacts with the Cys^{20} side chain located in the nucleotide-binding site of RhoC. A structure-based mechanism was proposed as an explanation for the formation of the RhoC-6-TGNP disulfide adduct caused by the reaction between 6-TGNP and the RhoC Cys^{20} side chain in the presence of a redox agent (Figure 5A). Mechanistically, the hooked 6-TGNP cannot be dissociated from RhoC by the RhoC GEF Dbs. This blockage of the Dbs-mediated 6-TGNP dissociation from RhoC disrupts the RhoC GTP/GDP cycle, resulting in downregulation of RhoC. Inactivation of RhoC leads to termination of the RhoC-induced cell motility of SUM149 and HME-RhoC cells. The proposed mechanism suggests that only the primary cysteine of the RhoC GXXXCGK(S/T)C motif plays a role in the formation of the 6-TGNP-RhoC adduct. Hence, it is predictable that the redox-sensitive GXXXXGK(S/T)C motif of Rac1 that also carries the primary cysteine can be a target of 6-TGNP to produce a Rac1−6-TGNP disulfide adduct.

Intriguingly, the action of 6-TGNP with a redox agent on RhoC yields a different result than the action of 6-TG with a redox agent on Ras GTPase.[33] Although 6-TGNP with a redox agent blocks the RhoC guanine nucleotide exchange, the combination enhances the Ras guanine nucleotide exchange. The proposed structure-based molecular mechanism for RhoC (Figure 5A) in combination with the proposed structure-based molecular mechanism for Ras[33] explains this difference. Distinctly different from the location of the side chain of the redox sensitive Cys^{20} of the RhoC GXXXCGK(S/T)C motif,[21] the side chain of the redox sensitive Cys^{118} of the Ras NKCD motif is remote from the sulfur atom of the bound 6-TGNP.[53,54] Hence, unlike with RhoC, the 6-TGNP radical formed by the reaction between 6-TGNP and a redox agent in Ras cannot react with the remote Cys^{118} side chain to produce a Ras−6-TGNP disulfide adduct. Instead, the 6-TGNP radical in Ras further reacts with another redox agent to produce a redox agent−6-TGNP adduct such as a NO_2−6-TGDP adduct, which is then released from Ras.[33] This comparative analysis also predicts that the GXXXCGK(S/T)C and GXXXXGK(S/T)C motif-containing Rho proteins—including RhoC and Rac, but not the NKCD motif-containing Ras GTPases—can be a target of 6-TGNP in the presence of a redox agent to produce a Rho GTPase−6-TGNP adduct. Hence, the targeting action of 6-TPs with a redox agent is likely specific for Rho GTPase, but not for Ras.

Role of a Redox Agent on the 6-TP-Mediated Termination of Invasive Motility of SUM149 and HME-RhoC Cells. A redox agent is necessary to produce a disulfide bond between 6-TGNP and RhoC *in vitro* (Figure 4A). The proposed mechanism (Figure 5A) suggests the role of the redox agent on formation of the RhoC-6-TGDP adduct. However, although treatment with a redox agent certainly enhances loss of the 6-TG-mediated cell motility of SUM149 and HME-RhoC cells and formation of the RhoC-6-TGNP disulfide adduct, this treatment was not indispensable to observing the 6-TP effect on SUM149 and HME-RhoC cells coupled with formation of the RhoC-6-TGNP disulfide adduct. It is likely that the action of 6-TPs on the motility of SUM149 and HME-RhoC cells is coupled with cellularly produced redox agents, such as $O_2^{\bullet-}$ and $^{\bullet}NO_2$ (a product of the reaction of NO with O_2).

The redox agent-mediated enhancement of the action of an antitumor agent is not unprecedented. One study has shown that NO enhances the antitumor activity of *cis*-diamminedichloroplatinum(II) (cisplatin) on HNSCC cells.[48] Cisplatin has been shown to mainly target DNA.[55] However, cisplatin also can react with the thiols of Cys^{16} and Cys^{20} of the RhoA GXXXCGK(S/T)C motif to form a cisplatin−RhoA adduct in the course of producing an inactive RhoA; a NO donor enhances the formation of this cisplatin−RhoA adduct.[20] Given that RhoC also possesses the GXXXCGK(S/T)C motif, it is possible to speculate that cisplatin can target RhoC to product a cisplatin−RhoC adduct. Both RhoA and RhoC have been shown to be expressed ubiquitously in HNSCC cells.[56,57] Hence, the redox-mediated enhancement of the formation of the cisplatin−RhoA and/or cisplatin−RhoC adduct remains one of many possibilities to investigate as contributors to the anticancer effects of cisplatin on HNSCC cells.[48] The lack of conclusive evidence notwithstanding, it is intriguing to note that the action of a NO donor on 6-TG and cisplatin with Rho proteins commonly results in a linkage that enhances the formation of thiol-related protein adducts.

Significance of the Action of 6-TG and a Redox Agent on IBC and Its Relevance to Other GTPases. Other Rho proteins that contain the GXXXCGK(S/T)C and GXXXXGK(S/T)C motifs, such as RhoA and Rac1, respectively, are also endogenously expressed in SUM and HME-RhoC cells, and these proteins play important roles in cellular signaling events.[58,59] However, because RhoC is mainly overexpressed in IBC cells among other Rho GTPases, the populated RhoC could be, by mass action, a primary target of 6-TPs. This analysis supports a hypothesis that 6-TPs with a redox agent may be an effective antimetastasis agent for IBC.

This study also showed that 6-TG with a redox agent can also target RhoC and terminate RhoC-mediated motility in other tumors such as SW480 and HCCLM3 cells. This broad RhoC-targeting action of 6-TG in combination with a redox agent would open the door to the possibility of using 6-TG with a redox agent as an antimetastasis agent to impede the RhoC-mediated invasive metastasis of various tumors other than IBC.

Two steps are necessary before 6-TG with a redox agent can be used as an antimestastasis agent. The first is to further investigate and confirm with clinical trials the antimestastic effect of 6-TG with a redox agent on tumors cells overexpressed with RhoC. The second step is to develop an effective method to deliver a redox agent to the target cells to optimize the potential antimetastatic effect of 6-TG on tumor cells overexpressed with RhoC. Although a delivery system exists for 6-TG and its analogues, no such method yet exists for redox agents.

Potential Effect of Glutathione Associated with a Redox Agent on 6-TG-Mediated Termination of Cell Motility. We have shown that a reducing agent like DTT (or β-mercaptoethanol) can liberate 6-TGNP from RhoC via reduction of the disulfide bond between 6-TGNP and RhoC (Figure 4D). As with DTT (or β-mercaptoethanol), a cellular redox buffer glutathione (GSH)[60] is known to reduce disulfides.[60,61] Therefore, although it could be dependent on an effective local cellular concentration of GSH, GSH may reductively break down the RhoC−6-TGNP disulfide adduct. Furthermore, GSH, in the form of glutathiolate, can react with various cellular redox agents (e.g., $^{\bullet}NO_2$ and $O_2^{\bullet-}$).[60,61] These

factors form the basis for speculation that cellular GSH impedes formation of the RhoC−6-TGNP adduct in cells via scavenging endogenously released or treated cellular redox agents. Such a GSH-mediated reductive split of the RhoC−6-TGNP adduct as well as blockage of formation of the RhoC−6-TGNP adduct could diminish the cellular content of the RhoC−6-TGNP adduct and thus negate the effect of 6-TG on the termination of the RhoC-mediated cell motility.

Notably, however, although 6-TPs can be converted into 6-TGNP, they also deplete cellular GSH.[62−64] Because of the diminution of GSH in cells, the potential antagonistic effects of GSH on the action of 6-TG in combination with a redox agent on cell motility may not be as extensive as expected. This notion is supported by our results showing that treatment of SUM149 and HME-RhoC cells with 6-TG and a redox agent was followed by detection of the RhoC−6-TGNP adduct and observation of the complete cessation of cell motility (Figure 1A).

Potential Application of These Findings and Further Studies. Our findings are significant because they open a possible route to the development of new drugs and/or a potential chemotherapeutic application of these old drugs as antimetastatic agents for IBC.

Notably, previous studies[28,34] have shown that in cells 6-TGNP derived from 6-TPs prefers to bind to Rac1 instead of to Ras; however, Rho GEF Vav is unable to facilitate the GNE of the Rac1-bound 6-TGNP with GTP. Our result explains these previously enigmatic observations in which the observed target selectivity of 6-TPs for Rac1 was likely the result of the reactivity of 6-TGNP (a metabolic product of treated 6-TP) with the Rac1 Cys^{18} (equivalent to RhoC Cys^{20}) to produce a 6-TGNP−Rac1 adduct. This adduct, in turn, blocked the action of Vav for Rac1 GNE.

Taken together, although further studies are required, our results appear to deliver the basis for a strategy to develop such agents for immune disorders as well as diseases of the heart, lungs, and other organs when such diseases are associated with dysregulation of other GTPases, such as RhoA and Rac,[6−14] respectively, containing the GXXXCGK(S/T)C and GXXXXGK(S/T)C motifs.[19,20]

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ABBREVIATIONS USED

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; 6-TG, 6-thioguanine; 6-TGDP, 6-thioguanosine diphosphate; 6-TGNP, 6-thioguanosine phosphate; 6-TGTP, 6-thioguanosine triphosphate; 6-TP, 6-thiopurine; $^{app}K_D$, apparent dissociation constant; cisplatin, *cis*-diamminedichloroplatinum-(II); 6-TdGNP, deoxy-6-thioguanosine phosphate; 6-TdGNP, Dbl's big sister; DETA/NO, diethylenetriamine/nitric oxide; N_2O_3, dinitrogen trioxide; DTT, dithiothreitol; ESI-MS, electrospray mass spectrometry; GSH, glutathione; GEFs, guanine nucleotide exchange factors; GNE, guanine nucleotide exchange; HNSCC, head and neck squamous cell carcinoma; IBC, human inflammatory breast cancer; HME-C20S RhoC cells, mutant C20S RhoC-overexpressed human mammary epithelial cells; NO, nitric oxide; $^{\bullet}NO_2$, nitrogen dioxide; GSNO, *S*-nitrosoglutathione; $O_2^{\bullet-}$, superoxide anion radical; MS/MS, tandem mass spectrometry; $^{true}K_D$, true dissociation constant; HME-RhoC cells, wild-type RhoC-overexpressed human mammary epithelial cells.

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