ABSTRACT

O6-Methylguanine-DNA methyltransferase (MGMT), a constitutively expressed DNA repair protein, removes alkyl groups from the O6-position of guanine in DNA. Tumor cells with high MGMT activity are resistant to nitrosoureas and other agents that form toxic O6-alkyl adducts. O6-Benzylguanine (BG) inactivates the MGMT protein and thereby enhances the sensitivity of tumor cells to alkylating drugs. However, the therapeutic potential of BG is limited by its poor solubility and its nonspecific inactivation of MGMT in normal tissues as well as in tumor tissues. Consequently, BG analogues are being developed to identify agents that have more favorable pharmacological characteristics.

We evaluated O6-benzyl-2'-deoxyguanosine (dBG), the 2'-deoxyribonucleoside analogue of BG, for its ability to inhibit MGMT and to potentiate 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) in a MGMT-positive human brain tumor xenograft, Daoy. When given i.p. 1 h before BCNU (25 mg/m2) to animals bearing s.c. tumors, dBG (134 mg/m2) produced a growth delay of 24.7 days, compared to 21.6 days after treatment with an equimolar dose of BG (90 mg/m2) plus BCNU and −0.6 days after treatment with BCNU alone. The combination of dBG + BCNU also increased the survival of animals bearing intracranial tumors by 65%. By increasing the dose of dBG to 300 mg/m2 (the maximum dose that could be delivered i.p. in a standard treatment volume), the growth delay of s.c. tumors increased from −0.1 days with BCNU alone to 39.3 days. dBG suppressed both tumor and liver MGMT activity to less than 1.5% of baseline, and dBG + BCNU induced extensive perivascular apoptosis.

Because dBG is a 10-fold less potent MGMT inhibitor than BG in HT-29 cell extracts, these results illustrate the capacity of BG analogues to potentiate BCNU toxicity, despite less in vitro activity than the parent compound, and emphasize the importance of in vivo evaluation of BG analogues.

INTRODUCTION

Drug resistance is a major problem in the chemotherapy of human tumors (1). One well-characterized mechanism of drug resistance involves the protein MGMT,2 which removes alkyl adducts from the O6-position of guanine in DNA (2). Nontoxic MGMT inhibitors significantly increase the sensitivity of tumors to the cytotoxic effects of agents, such as the nitrosoureas, procarbazine, temozolomide, and others, that form O6-alkylguanine DNA adducts.

The lead compound being developed as an MGMT inhibitor is BG (3). It has a high affinity for MGMT, and the reaction of BG with MGMT inactivates the repair protein, thereby enhancing the cytotoxicity of nitrosoureas and methylating agents (4). However, its solubility in clinically acceptable formulations is limited, and its effects are nonspecific in that MGMT is inhibited in tumor and normal tissues alike.

Numerous BG analogues have been synthesized in an effort to find and develop one with more favorable pharmacological characteristics (5). One such compound, dBG, the 2'-deoxyribonucleoside analogue of BG, is of interest because of its greater solubility and anticipated differences in its pharmacology, although the in vitro screen using HT-29 cell extracts indicated that it was approximately 10-fold less potent than BG as an MGMT inhibitor. In this study, we report the evaluation of dBG as an in vivo inhibitor of MGMT and as a BCNU-potentiating agent in a human brain tumor xenograft in athymic mice.

MATERIALS AND METHODS

Chemicals. BG and dBG were synthesized and purified according to methods published previously (4, 6, 7). [3H]MNU (specific activity, 18 Ci/mmol) was purchased from Amersham (Arlington, IL). BCNU was purchased commercially.

Animals. BALB/c nu/nu athymic mice at least 6 weeks old and derived from an independent breeding colony at University of Texas Southwestern were used in all experiments. Mice were maintained under barrier conditions and given sterilized food (Harlan Teklad Laboratory Diet) and water.

Tumor Line. Daoy, a hypertetraploid human medulloblastoma cell line (8), grows s.c. in athymic mice with a doubling time of 4.9 days. Its untreated growth rate reached 500 mm3 before treatment. Tumors were measured in two perpendicular dimensions, and their volumes were estimated by the formula

\[ V = \frac{4}{3} \pi r^3 \]

where \( r \) is the radius. Tumor volumes were recorded weekly, and tumor regression was defined as a tumor volume smaller than the starting volume on the treatment day.

Drug Treatments. All treatments were administered i.p. at a volume of 30 ml/m2. BG was given at a dose of 90 mg/m2, and dBG was given at doses of 134, 200, or 300 mg/m2. BG and dBG were dissolved in 40% PEG 400 in phosphate buffer (0.05 M; pH 8.0). BCNU was administered at a dose of 25 mg/kg in 10% ethanol in water. BCNU, or its solvent, was administered 1 h after BG, dBG, or the 40% PEG solvent. Although dBG is water soluble, it was dissolved in 40% PEG for these experiments to make the results comparable to those achieved with BG.

Treatment of s.c. Tumors. These experiments were done using standard procedures that have been described elsewhere (9, 10). In brief, 50 μl of tumor were implanted in the right flank of animals. Tumors were sequentially measured in two perpendicular dimensions, and their volumes were estimated using the formula

\[ V = \frac{4}{3} \pi r^3 \]

where \( a \) is the shorter and \( b \) the longer of the dimensions. Tumors were measured twice weekly until their volumes exceeded five times their volume at treatment. The data were analyzed using the Wilcoxon rank sum test, comparing the time from treatment to five times treatment volume in each of the groups. The growth delay was the difference between the median time to five times treatment volume in the treatment group minus the median time to five times treatment volume in the control group. In addition, the number of tumor regressions occurring in each group was determined, and these groups were compared by the two-tailed Fisher exact test. Tumor regression was defined as an any tumor volume smaller than the volume on the treatment day.

Two treatment experiments were done using s.c. tumors. In the first, the ability of equimolar doses of BG and dBG to potentiate the antitumor activity of BCNU was compared using four groups of mice. The two treatment groups received BG (90 mg/m2) or dBG (134 mg/m2) plus BCNU. The two control groups received vehicle (40% PEG, followed in 1 h with 10% ethanol) or 40% PEG + BCNU. In the second experiment, three groups of mice were used to determine the efficacy of the maximum soluble dose of dBG in this volume of solution (300 mg/m2) in potentiating BCNU. The three treatment groups

Received 11/16/95; accepted 3/4/96.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported in part by NIH Grant CA 57725.
2 To whom requests for reprints should be addressed, at Department of Neurology, University of Texas Southwestern Medical Center at Dallas, Dallas, TX 75235-9036.
3 The abbreviations used are: MGMT, O6-methylguanine-DNA methyltransferase; dBG, O6-benzyl-2'-deoxyguanosine; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; MeG, methylguanine; i.e., intracranial; PEG, polyethylene glycol; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.
were the drug vehicle, 40% PEG + BCNU, and dBG + BCNU. Between 10 and 16 animals were used in each treatment group in these experiments.

Treatment of i.e. Tumors. Treatment experiments in mice bearing i.e. tumors were performed using standard procedures (10). In brief, 5 µl of a tumor suspension were implanted into the right cerebral hemisphere of anesthetized animals. Thirty-one days after tumor implantation, seemingly healthy animals as determined from weight and gross appearance were randomly divided into three treatment groups of 10–14 animals. The treatment groups were drug vehicle (40% PEG + 10% ethanol), 40% PEG + BCNU, and dBG (200 mg/m³) + BCNU. Animals were followed daily until death. Survival times following treatment were compared among the groups using the Wilcoxon rank sum test.

MGMT Assay. MGMT activity was determined in tumors and livers of mice at various times after administration of 300 mg/m² dBG. Treated animals were anesthetized with methoxyflurane and perfused via the heart (left atrium) with warm (35°C) PBS-0.1 mM EDTA for 5 min to remove the unmetabolized drug and metabolites that could interfere with the assay due to their reactivity with MGMT. After perfusion, the tumor and livers were removed and kept at −80°C until used. Tumors were trimmed of necrotic tissue and blood clots and were cut into fragments of approximately 20 mm³, weighed, and homogenized in three volumes of 50 mM Tris-HCl, 0.1 mM EDTA, and 2 mM DTT (pH 7.5; homogenizing buffer) with a Teflon homogenizer. Homogenates were placed in microcentrifuge tubes, sonicated for 10 s (three times) at 70% maximum output, and centrifuged at 15,000 × g for 15 min. Supernatants were removed, and their protein concentration was determined according to Bradford (11). Livers from tumor-bearing animals were also excised, minced, and homogenized with an E5SE biotomerogenizer (Bartlesville, OK) in three volumes of homogenizing buffer for 30 s at low speed and 10 s at high speed. Homogenates were centrifuged at 15,000 × g for 20 min, and the supernatants were removed and stored at 0°C. Pellets were rehomogenized in two volumes of homogenizing buffer, sonicated for 10 s (three times), and samples were centrifuged at 15,000 × g for 15 min. The supernatants of the two centrifugations were combined, and their protein concentrations were determined; then the supernatants were stored in liquid nitrogen until further use. All tissue handling and procedures were done at 0°C.

The MGMT activity assay of livers and tumors was determined according to a modification of methods published previously (12). Briefly, DNA, which had been methylated with [3H]MNU and contained 60 fmol of $^{3}$H-MeG (specific activity, 16.5 ± 0.5 Ci/mmol), was incubated with varying volumes of tumor or liver MGMT extracts for 1 h at 37°C. The final volume of all incubations was adjusted to 500 µl with homogenization buffer. Reactions were quenched with 0.1 N HCl, and samples were incubated for an additional 45 min at 70°C. Samples were cooled on ice for 1 h and centrifuged at 14,000 × g for 5 min; then the supernatant was removed and neutralized with sodium bicarbonate and dried by lyophilization. Lyophilized samples were dissolved in 0.12 ml 0.1 N HCl and centrifuged at 14,000 × g; then adducts were separated by high-performance liquid chromatography, according to published procedures (13). $^{3}$H-MeG:7-MeG ratios were determined at varying protein concentrations from each sample (usually four) and plotted against such concentrations. The intercept of the central linear response of the curve (ratios between 0.9 and 0.3) with the X axis marked the amount of total protein in the sample required for the removal of all 60 fmol of $^{3}$H-MeG from the substrate. For samples having MGMT activity less than 20 fmol/mg protein, determinations were repeated using only one-third of the substrate. The method is sensitive enough to detect 5 fmol/mg protein with a standard deviation not exceeding 15%.

In Vivo Determination of Cell Death in Tumors. To identify treatment-related apoptotic cell death, animals bearing i.e. Daoy tumors were treated with either drug vehicle, BCNU alone, or dBG (134 mg/m²) + BCNU. Tumors from the animals treated with dBG + BCNU were removed for analysis on days 0 (prior to treatment), 1, 3, 7, 12, and 19. Tumors from the control and BCNU alone groups were removed on days 0, 1, and 3 only since tumor growth was unaffected by treatment, and the animals had to be euthanized. Tumors were divided, fixed in 10% buffered formalin for 24 h, sectioned, embedded in paraffin, and step-sectioned at 5 µm at 20–µm intervals. One set of histological slides was stained with H&E to evaluate tumor necrotic areas and mitoses. Apoptosis was determined in situ on another set of slides using the Oncor ApopTag kit (Oncor, Gaithersburg, MD). According to the method, residues of digoxigenin-nucleotide are catalytically added to the DNA by terminal deoxynucleotidyl transferase, which catalyzes a template-independent addition of deoxyribonucleotide triphosphate to the 3'-OH ends of DNA. The incorporated nucleotides form random heteropolymers of digoxigenin-11-dUTP and dATP. The anti-digoxigenin antibody fragment carries conjugated peroxidase as a reporter to the reaction site, which then catalytically generates an intense signal from chromogenic substrates. Random DNA breaks, such as those developed by depurination following BCNU treatment, are not sufficient to yield a intensely distinct signal, such as that manifested during the apoptotic process.

RESULTS

s.c. Treatment Efficacy. The median times from treatment to five times the tumor volume at treatment were 9.6 and 9.0 days, respectively, in vehicle-treated control mice and mice treated with BCNU alone. This difference was not significant. On the other hand, mice treated with BG + BCNU had a median time to five times treatment volume of 31.2 days. This corresponds to a growth delay of 21.6 days. Similarly, mice treated with dBG (134 mg/m²) + BCNU had a median time to five times treatment volume of 34.3 days, corresponding to a growth delay of 24.7 days. Although the difference between BG and dBG was not statistically different (P = 0.175), the combination treatment with either MGMT inhibitor and BCNU significantly inhibited tumor growth as compared to both control and BCNU alone groups (P < 0.0001; Table 1; Fig. 1). Furthermore, tumor regressions were not detected in either the control or the BCNU alone groups, whereas there were three regressions in the 16 animals receiving BG + BCNU and nine regressions in the 16 animals receiving dBG + BCNU. The difference between BG and dBG in producing regressions was marginally significant (P = 0.068).

The effect of a higher dose of dBG was examined using 300 mg/m², which was the maximum possible dose based on the solubility of the drug in this solvent at the treatment volume of 30 ml/m². In this experiment, the median time from treatment to five times treatment volume was 7.9 days in vehicle-treated control animals and 7.8 days in animals treated with BCNU alone. Animals treated with dBG + BCNU had a median time to five times treatment volume of 47.2 days, which corresponds to a growth delay of 39.3 days. This value was significantly different from both control animals and those receiving BCNU alone (P < 0.0001; Table 2; Fig. 2). Furthermore, of the tumors in 12 animals receiving dBG + BCNU, 11 regressed and the 12th animal died one day following treatment (before tumor regression would have been detected). This was highly significantly different from 0 of 10 regressions in both the vehicle-treated control group and the PEG + BCNU group (P < 0.0001; Table 2).

l.c. Treatment Efficacy. The median survival of mice with implanted Daoy i.e. was 10 days following treatment, and this was not affected by BCNU alone. However, median survival increased to 16.5 days in mice treated with dBG (200 mg/sq2) + BCNU (Table 3; Fig. 3). This represents 65% increase in the life span of the animals that were treated with the combination of dBG and BCNU, compared to those treated with BCNU alone (P < 0.0001).

Table 1 Treatment of s.c. Daoy xenografts in athymic mice with equimolar doses of BG or dBG + BCNU

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of animals</th>
<th>Median time to 5X T/C (days)</th>
<th>P vs. control</th>
<th>Tumor regressions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug vehicle</td>
<td>10</td>
<td>9.6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BCNU</td>
<td>10</td>
<td>9.0</td>
<td>0.6</td>
<td>NS&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>BG + BCNU</td>
<td>16</td>
<td>31.2</td>
<td>21.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>dBG + BCNU</td>
<td>16</td>
<td>34.3</td>
<td>24.7</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

<sup>a</sup> Drug doses: BCNU, 25 MG/m²; BG, 90 MG/m²; dBG, 134 MG/m². All drugs were administered i.p. at a volume of 30 ML/m².
<sup>b</sup> Median survival in treatment group/median survival in control (drug vehicle) group.
<sup>c</sup> NS, not significant.
than that observed in untreated tumors or in the rest of treated tumor. However, such areas were microscopic and not present in all sections. In contrast to untreated tumors and tumors treated with BCNU alone, apoptotic and preapoptotic (cells staining but not yet having morphological features of apoptosis) bodies were common in tumors of animals following dBG + BCNU treatment (Fig. 5). A significant increase of focal apoptosis was seen 3 days after dBG + BCNU. More extensive apoptotic death, mainly around blood vessels, was seen on day seven, before regression of the tumor mass. However, a much larger number of true apoptotic bodies and fragmented nuclei were visible in regressing tumors 12 and 19 days after treatment (Fig. 5). Extreme apoptosis and nuclear fragmentation were also observed in necrotic areas of regrowing tumors, 3 or more weeks after treatment (data not shown).

**DISCUSSION**

MGMT is an important protein that contributes to the resistance of human tumors to the cytotoxic effects of agents that alkylate DNA at the N\(^\alpha\)-position of guanine. It has been shown repeatedly that MGMT activity correlates with the resistance of human tumor xenografts to BCNU, procarbazine, and related drugs (16, 17). Since most human neoplasms contain substantial levels of MGMT activity and are, therefore, resistant to many alkylating drugs, mechanisms for inhibiting MGMT activity would have important implications for overcoming drug resistance and achieving therapeutic success.

BG has been shown to be a potent MGMT inhibitor and to potentiate the therapeutic effect of BCNU and related agents against human tumor xenografts (4, 9, 10, 18). Its importance is increased by the fact that it is relatively nontoxic alone. Its principal limitations appear to be its solubility and its potentiation of the toxic effects of alkylating

---

**Table 3 Treatment of i.e. DAOY xenografts in athymic mice with dBG + BCNU**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of animals</th>
<th>Median survival after treatment(b) (days)</th>
<th>P vs. control</th>
<th>50 day survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug vehicle</td>
<td>11</td>
<td>10</td>
<td>1.00</td>
<td>0</td>
</tr>
<tr>
<td>BCNU</td>
<td>12</td>
<td>10</td>
<td>NS(d)</td>
<td>0</td>
</tr>
<tr>
<td>dBG + BCNU</td>
<td>14</td>
<td>16.5</td>
<td>&lt;0.0001</td>
<td>2</td>
</tr>
</tbody>
</table>

\(a\) Drug doses: BCNU, 25 MG/m\(^2\); dBG, 200 MG/m\(^2\). Drugs were administered i.p. at a volume of 30 ML/m\(^2\).

\(b\) Animals were treated 31 days following tumor implantation.

\(c\) Median survival in treatment group/median survival in control (drug vehicle) group.

\(d\) NS, not significant.
drugs on normal tissues. Nevertheless, O6-BG has been developed by the National Cancer Institute as a potential clinical agent and has entered clinical trials within the last year.

BG was synthesized as a potential MGMT inhibitor following the initial observation that a variety of O6-alkylguanines were substrates for the MGMT protein (3). BG was subsequently found to strongly potentiate the cytotoxic effects of BCNU (19). In an in vitro screen of potential MGMT-inhibiting BG analogues, dBG was found to be 10-fold less potent than BG and BGS modified at the benzyl ring, but it was among the most active of the relatively soluble MGMT inhibitors (5). The relatively high potency of dBG combined with its superior solubility in aqueous media compared to the BGS prompted us to test it in an in vivo xenograft system.

In the experiments reported here, we have demonstrated that, despite the substantial difference in in vitro potency between BG and dBG, the BCNU-potentiating effects of the two compounds were quite comparable. Furthermore, escalation of the dBG dose was not restricted by solubility, as was the case with BG, and therefore, it was possible to achieve a much greater potentiation of BCNU with dBG than could be achieved with BG. In addition, the BCNU-potentiating effect of dBG could be demonstrated with the tumor growing in the brain, as had been the case with BG (10).

This potentiation was achieved without any significant toxicity to the tumor-bearing animals. There was treatment-related mortality and significant weight loss only in the animals treated with the maximum soluble dose of dBG + BCNU. The dose of BCNU was substantially lower than the animals could have tolerated without BG or dBG, but we have shown that even at the LD₅₀ of BCNU, this human brain tumor xenograft (Dazy) is highly resistant to BCNU (8). There can be no doubt that these MGMT inhibitors have converted a tumor that in its native state is highly resistant to BCNU to a sensitive tumor, without substantial toxicity.

The presumed mechanism of this sensitization is inhibition of MGMT. No other plausible hypothesis for potentiation of BCNU (and related alkylating agents) has been proposed for these BG analogues, and it is clear that dBG is a potent inhibitor of MGMT. We found maximum inhibition 8 h after dBG administration, and therefore, it is possible that even greater therapeutic potentiation could be achieved if the interval between dBG and BCNU were increased. Other treatment schedules (e.g., multiple smaller doses) or different formulations might also be more effective in potentiating the cytotoxic effects of a single dose of BCNU, but we made no attempt to assess that in these experiments. Our data also suggest that tumor and certain normal organs might resynthesize MGMT at a somewhat different rate (Fig. 4); therefore, it might be possible to enhance the differential effect on tumor and normal organs by timing the BCNU administration to coincide with relative normal organ recovery and continued tumor MGMT suppression.

We believe that the discrepancy between the relative potencies of BG and dBG in in vitro and in vivo systems is due to the extensive metabolic conversion of dBG to BG and O6-benzyl-8-oxo-guanine (20, 21). Greater solubility and possibly systemic availability of dBG compared to BG may result in greater or more prolonged intracellular levels of BG in dBG-treated tumors than in those treated with BG alone. Although we do not yet have an enzymatic explanation for the loss of the sugar moiety, it is clear that it occurs to an extent that could produce substantial MGMT suppression. The conversion from dBG to BG continues over several hours, probably accounting for continued MGMT suppression, despite the relatively short plasma half-life of the administered compound. In addition, the deoxynucleoside may be transported into cells to a greater extent than the base, and this could account for some of the initial MGMT suppression. These results emphasize the limitation of in vitro screening of MGMT inhibitors, particularly if there are significant metabolic differences between an analogue and the parent compound.

Apoptosis is a mode of cell death characterized by distinctive biochemical and morphological features that are regulated by several genes, including bcl-2, c-myc, and p53 (22–24). Recent evidence suggests that apoptosis plays an important role in the response of tumors to various forms of therapy (23, 25). There may be two pathways leading to apoptosis as an effect of anticancer therapy: one involving G₂M cells and another that is cell cycle independent (26). The former pathway leads to delayed induction of apoptosis, whereas the latter produces a more rapid effect. For example, tumor cells treated with taxol undergo apoptotic death only when they are arrested in mitosis (27), whereas radiation leads to a rapid, cell cycle-independent apoptosis (28). In the system used in the experiments described in this study, we have induced apoptosis with dBG + BCNU that reaches a peak 2 weeks after treatment. This is later than the apoptotic events usually observed after radiotherapy or chemotherapy of tumors. This may be due to the relatively low dose of BCNU used in these experiments (26) or to the specificity of the DNA damage produced by the dBG-BCNU combination. It remains unclear how the cross-links that are produced by BCNU when MGMT activity has been eliminated are related to apoptotic cell death. The limited results
Fig. 5. Cell death in s.c. Daoy tumors in athymic mice after treatment with dBG (134 mg/m²) + BCNU (25 mg/m²). Extensive cell death, as determined by TUNEL, was observed 13 days after treatment. Dead or dying cells were found mainly in the proximity of blood vessels (A; ×200). Apoptotic bodies characterized by large chromatin masses surrounded by a thin cytoplasmic rim and single condensed nuclei or condensed nuclear fragments were found scattered among live cells (C; ×400). Continuous cell death and loss of vascular integrity, and observed 17 days after dBG/BCNU treatment, coincided with tumor regression (B; ×200). Apoptosis, as evidenced by TUNEL and morphological criteria, is the major mode of cell loss at this stage. Large numbers of mast cells are found among apoptotic bodies (D; ×400). All slides were stained with methyl green.
shown here only demonstrate that apoptosis occurs in this particular tumor, and it seems to play some role in its response to dBG + BCNU. The large accumulation of apoptotic bodies adjacent to blood vessels and subsequent collapse of such vessels demonstrates that one or both drugs have a limited capacity to diffuse from the vasculature within the tumor.

These results imply that additional MGMT-suppressing compounds that are highly potent drug resistance modulators and possess favorable pharmacological characteristics might be identified. It is also not unreasonable to speculate that compounds that have differential MGMT-suppressing effects on tumor and normal organs might be developed.

REFERENCES


Downloaded from cancerres.aacrjournals.org on April 14, 2017. © 1996 American Association for Cancer Research.
Treatment of Human Brain Tumor Xenografts with \(O^6\)-Benzyl-2′-deoxyguanosine and BCNU

S. Clifford Schold, Jr., Demetrius M. Kokkinakis, Jack L. Rudy, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/56/9/2076

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.