Mechanism of Action of Bisimidazoacridones, New Drugs with Potent, Selective Activity against Colon Cancer

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ABSTRACT

Antitumor bisimidazoacridones are bifunctional DNA binders which have recently been shown to selectively target human colon carcinoma cells in vitro and in vivo and appear to be excellent candidates for clinical development. We have studied the mechanism of action of one bisimidazoacridone, WMC26, which is 1,000–10,000 times more toxic to human colon carcinoma cells (HCT116) than to melanoma cells (SKMEL2) in vitro. Plasmid DNA exposed to WMC26 showed enhanced digestion by DNase I at A-T-rich sites, suggesting alterations in DNA conformation upon drug binding. These results led us to investigate whether WMC26 was selectively toxic due to a specific recognition of DNA bends by repair excinucleases, as has been demonstrated with the DNA bisintercalator, ditercalinium. Both prokaryotic and eukaryotic cells with intact repair capacity were shown to be selectively sensitive to WMC26, strongly indicating that excision repair plays a role in its toxicity. Confocal microscopy studies utilizing fluorescence of the WMC26 chromophore showed compound localization in the perinuclear cytoplasmic area, as had been previously noted for ditercalinium, indicating that cytoplasmic DNA could be the target. This irreversible accumulation of compound was gradually followed by vacuolization of the cytoplasm and cell death. Cell cycle analysis of both lines treated with WMC26 or with ditercalinium showed that, while the latter induced HCT116 growth arrest at G1–G2, WMC26 also blocked the cell cycle at G1–M; SKMEL2 cells did not undergo any changes in cell cycle as a result of either treatment. Our data show that WMC26 is 10–100 times more cytotoxic than ditercalinium in vitro. Like ditercalinium, WMC26 appears to exert its toxicity via cytoplasmic elements, through a mechanism involving excision repair processes. However, its highly selective cytotoxicity may stem from additional undefined targets in sensitive colon cancer cells.

INTRODUCTION

Many antitumor drugs interact with DNA either by covalent bond formation, such as that observed with alkylating agents, or by non-covalent binding. The latter group includes agents which form tight complexes with either the major or minor grooves of B-DNA, or those which intercalate between bp of the double helix. Intercalation into DNA is a common feature of many important antitumor agents (1, 2). For example, the anthracycline drugs such as doxorubicin or daunomycin, which are known intercalators, have several demonstrable biological activities, such as the inhibition of topoisomerase II, but ultimately cause the inhibition of nucleic acid synthesis and cell death (3). The cytotoxicity of some agents has been correlated with their propensity for intercalation (4), but there are a number of effective intercalators which are devoid of cytotoxic or antitumor activity. This suggests that the ability to intercalate may be a useful adjunct property of some drugs, but that intercalation on its own is not sufficient for activity (5). The effect of intercalation can be enhanced if a drug contains two moieties which are able to insert between bp. A number of DNA bisintercalators have been synthesized whose ability to bind to DNA rivals that of some regulatory proteins (6). It has been demonstrated that the cytotoxicity of some of these agents correlated with their enhanced binding to DNA, relative to the monomers. However, this is not always the case, as shown by the lower activity of a bisdaunomycin derivative relative to the corresponding monomer, even though the bisintercalator bound much more tightly to the DNA (7). Similarly, diacridines were shown to be excellent bisintercalators, but this property alone did not translate into useful drugs (1, 8, 9). Thus, bisintercalation, just like monointercalation, does not in itself impart antitumor properties. Since bisintercalators occupy larger segments of the DNA than their monointercalator counterparts, it is possible that sequence selectivity may play an important role in the determination of biological activity through, for example, inhibition of gene expression or other effects induced by pronounced changes in DNA conformation (10, 11).

We have recently reported (12) on the discovery of a new class of potential bisintercalators, the bisimidazoacridones. These compounds were found to be DNA binders with potent, broad spectrum activity in vitro as well as extremely high selectivity for colon tumors in vitro and in vivo. In spite of the intent to prepare a new class of bisintercalators, preliminary data suggest that these compounds do not intercalate into DNA, but rather bind in the grooves. Drug selectivity based on differential accumulation in the mitochondria of carcinoma versus normal cells has been discussed previously by Nadakavukaren et al. (13). In this article we report on studies of the mechanism of action of one of the most promising of the bisimidazoacridones (Fig. 1). The compound, in common with its congeners, localizes in the cytoplasm of tumor cells, and induces a slow toxic process during which mitochondrial DNA appears to become degraded. The data suggest that the compound induces a DNA distortion which is recognized by repair enzymes, and the repair process does not proceed normally. This effect produces an irreversible growth arrest which results in cell death. Similarities and differences between the bisimidazoacridones and the authentic bisintercalator, ditercalinium, are discussed.

MATERIALS AND METHODS

Chemicals and Materials. All chemicals, bacterial culture reagents, and mammalian cell culture reagents were from Sigma. Tissue culture and bacterial culture materials were from Costar. Ditercalinium was a generous gift from the American Type Culture Collection. The synthesis of bisimidazoacridones has been described previously (12). Plasmid pEUK1 was from Clontech and restriction enzymes were from New England Biolabs. [γ32P]ATP was from Amersham and DNAse I footprinting reagents were from Pharmacia.

Colony Survival Assay. HCT116, SKMEL2, and XP16BE cell lines were obtained from the American Type Culture Collection and grown in DMEM containing 10% fetal bovine serum and standard antibiotics. Cells were maintained at 37°C in 5% CO2 in complete humidity. Assay cultures consisted of seeding 100–200 cells/well in 6-well plates and allowing them to attach for

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Experiments were conducted according to NCI in X-OMAT film. DNA was exposed to different concentrations of compounds in vitro at room temperature, immediately digested for 3 min with DNase I, and separated in agarose gels following complete digestion of methanol:PBS (v/v) of increasing methanol concentration and stained with methylene blue to show any effect. Since the median growth inhibition to tumor cells was 50% at 0.1 μM and 100 μM, respectively, and for this reason, we were allowed to grow at 37°C in the presence or absence of multiple concentrations of each drug. After 2 h, diluted samples of each culture were observed daily for 1 to 2 weeks, cells were fixed by a series of solutions of methanol:PBS (v/v) of increasing methanol concentration and stained with 1% crystal violet in methanol as described (14). Colonies of greater than 30 cells were scored as survivors.

**Bacterial Cytotoxicity Studies.** Escherichia coli strains AB1157 (wild type), AB1886 (UvrA mutant), and AB1884 (UvrC mutant) have been described previously (15). Briefly, cells were grown in 96-well plates and exposed to multiple concentrations of drugs continuously for 48 h. Drug-treated cultures were then stained with sulforhodamine B and assessed for growth inhibition and cytoxicity, based on comparisons to untreated controls. The colon tumor cell line subpanel included Colo205, HCC2998, HCT116, HCT15, HT29, KM12, and SW620; the melanoma cell line subpanel included Malme-3M, M14, SKMEL2, SKMEL28, SKMEL5, UACC257, and UACC-62.

**Confluent Microscopy Studies.** Cells were seeded in 4-well chamber slides and allowed to attach for 24 h. Compounds were added directly from stocks in DMSO or water in μM amounts to dishes and incubated for 2 h. Cells were then rinsed and fresh medium added. At different times, 10⁶ cells from each dish were removed with trypsin (14), washed twice by centrifugation with PBS containing 1% fetal bovine serum in the cold, and resuspended in 0.3 ml of the same solution. Cell fixation and staining for fluorescence-activated cell sorting was performed according to standard procedures (19). Briefly, 0.7 ml of ice-cold 100% ethanol was added dropwise, with vortexing, to cell suspensions, followed by two washes in PBS with 10% fetal bovine serum. Fixed cells were then treated with Rnase (1 unit/10⁶ cells) for 30 min at 37°C, chilled in ice, and stained with propidium iodide (50 μg/10⁶ cells) in the cold overnight. Fluorescence histograms of 10⁶ cells were obtained in a Coulter EPICS753 Cell Sorter with an argon laser and mean peaks analyzed.

**RESULTS**

As shown in Fig. 2, all seven colon tumor lines in the NCI cancer screening panel showed pronounced growth inhibition when treated for 48 h with WMC26, with median values of concentration needed to inhibit growth of 50% of cells in the 0.1–0.01 μM range. In contrast, all seven melanoma cell lines showed remarkable resistance to growth inhibition by the compound, requiring much higher concentrations (median concentration needed to inhibit growth of 50% of cells, 100 μM) to show any effect. Since the median growth inhibition to tumor cell lines from other tissues in the panel centered around 1 μM concentrations (12), it is clear that WMC26 shows great selectivity for colon adenocarcinoma cells. This effect was consistent with the results of our in vivo treatment of HCT116 xenografts, as shown in Table 1. Treatment with three doses of 25 and 50 mg/kg WMC26 shortly after tumors were measurable (day 8) produced optimal tumor weight changes of 39% and 14% of controls, respectively.
Examination of tissue sections from xenografts by CLSM showed compound fluorescence detectable for several days after the last dose (Fig. 3), and xenografts from treated animals exhibited considerably more fibrous encapsulation and extensive cytoplasmic vacuolization when compared to controls (data not shown) after 33 days postimplantation. Generally, WMC26-treated animals continued to gain weight normally throughout the experiment (Table 2) and showed minimal to mild signs of toxicity (on a severity scale ranging from minimal, to mild, to moderate, to marked), that being limited to lung mineralization and stomach hyperplasia, hyperkeratosis, and inflammation.

Our assessment of colony survival of HCT116 and SKMEL2 cells following a 2-h exposure to WMC26 and WMC25 (Fig. 4) showed that the latter was only mildly toxic (LC50 about 500 μM) to both cell lines, while WMC26 killed half the HCT116 cells at nm concentrations, and required μM concentrations for the same effect in SKMEL2 cells. Thus, WMC26 had the most appropriate structural features for profound, selective cytotoxicity to colon carcinoma cells, and it is likely that its growth inhibitory effect to the same cells was the reason for this toxicity.

When both WMC25 and WMC26 were allowed to react with DNA in vitro, WMC26-bound DNA showed enhanced digestion by DNase I in A-T-rich sequences (Fig. 5). This occurred at 1 μM concentrations of WMC26, while WMC25 was inactive even at 100 μM concentrations. This strongly suggested that the structure of WMC26 was better suited to a type of binding which induced bending of DNA in regions that favored digestion by DNase I. This effect correlated with the relative cytotoxicities of the two compounds to HCT116 cells.

In order to see whether WMC26 induced DNA structural changes recognizable by other nucleases which could be involved in its selective toxicity, we exposed E. coli strains differing in excision repair enzyme capacity to WMC25 and WMC26 during log phase growth and assessed their relative toxicities. Fig. 6 shows that WMC26 was over 10-fold more toxic to the AB1157 (repair-proficient) strain than to the AB1886 and AB1884 strains, which are severely deficient in UvrA and UvrC enzyme activities. WMC25, in turn, was mildly and equally toxic to all strains (nm concentrations). These results suggest that in bacteria, WMC26 but not WMC25 binds noncovalently to DNA and induces conformational changes which are preferentially recognized by enzymes involved in excising bulky DNA lesions,
resulting in an enhanced but ineffective cleavage of DNA which is probably related to cytotoxicity.

Since mammalian tumor cells are known to vary greatly in different DNA repair processes (20), we investigated whether a similar process could be responsible for the widely different toxicities of WMC26 to the various tumor cell lines. Fig. 7 shows the results of simultaneous exposure to the WMC compounds by cell lines HCT116, SKMEL2, and XP16BE (derived from a patient diagnosed with xeroderma pigmentosum, exhibiting 10–20% of normal UV-induced unscheduled DNA synthesis). The colony survival of SKMEL2 and XP16BE cells was similar after exposure to WMC26 (LC50 about 10 and 100 μM, respectively) while HCT116 cells were killed at nM concentrations. WMC25 was again equally but mildly toxic to all three cell lines. Fig. 8 shows the colony survival of the same cell lines after exposure to various doses of UV irradiation; XP16BE cells were killed at 10-J/m² doses, SKMEL2 cells were killed at doses nearing 30 J/m², and HCT116 cells survived doses up to 50 J/m². Together these experiments indicated that HCT116 cells had the greatest capacity to repair UV damage, and this may play a role in sensitivity of such cells to small doses of WMC26 and explain the relative resistance of melanoma cells to this compound.

Our observation of WMC26-specific fluorescence in live cells by CLSM is illustrated in Fig. 9. We found that the drug is readily internalized by both HCT116 and SKMEL2 cells, and after 1 h all detectable (green) fluorescence was in the perinuclear cytoplasmic areas and not in the nucleus, even after extensive washing. This showed that, once internalized, the compound was not easily expelled from cells. After 24–48 h, Nomarski imaging of HCT116 cells showed vacuolization of the cytoplasm, particularly in areas of heavy compound accumulation, as indicated by the specific green WMC26 fluorescence overlayed on the Nomarski image. Parallel treatment of HCT116 cells with ditercalinium, a fluorescent mitochondrial marker, showed similar cellular distribution and localization (data not shown). SKMEL2 cells also retained compound fluorescence, but only after 72–96 h could cytoplasmic vacuolization be seen. All cells were treated with 1 μM concentrations of WMC26, as this was a common toxic concentration for both cell lines (Ref. 12 and this study). These
drug concentrations. Like most bifunctional compounds of this type, these drugs bind strongly to DNA \textit{in vitro} (12), and our DNase I footprinting experiments indicate that the highly active WMC26, and not its close analogue WMC25, specifically alters DNA conformation such that enzyme digestion of specific A-T sequences is enhanced upon binding. DNase I cleavage of A-T-rich sequences has been experiments suggested that once toxic conditions are achieved, both cell lines showed similar morphological signs of injury; furthermore, they suggested that nuclear DNA may not be the primary cellular target of WMC26. Ditercalinium, a bisintercalator with structural similarities to the WMC compounds, has been shown to primarily target mitochondrial DNA (21).

HCT116 and SKMEL2 cell cycle analyses during exposure to WMC26 and ditercalinium (Fig. 10) showed a greater number of HCT116 cells in G2-M after 6–24 h of treatment with WMC26 (1 μM), a nearly total suppression of cells in S-phase, and a slight increase in G1-G0 cells, which indicated an arrest at this stage also. By 48 h, S-phase cells began to appear again and G2-M cells returned to control levels. Ditercalinium treatment (1 μM) induced only an increase in cells in G1-G0 by 24 h, a large decrease in S-phase cells and this effect was maintained by 48 h. Neither compound had detectable effects on cycle distribution in SKMEL2 cells for 48 h, again suggesting that μM concentrations are not sufficient for triggering the growth inhibitory effect observed in HCT116 cells at very early exposure times.

DISCUSSION

Our studies show that certain bisimidazoacridones are potent, irreversible growth inhibitors of human colon carcinoma cells by a process that results in specific toxicity to such cells at very low (nm)
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Fig. 10. Cell cycle analysis of HCT116 (A) and SKMEL2 (B) cells after treatment with WMC26 or ditercalinium. G, G1-G0; S; M. Cells were harvested at 6, 24, or 48 h after initial treatment with WMC26 or ditercalinium, or no treatment. Cell percentages are averages of three separate samples, analyzed as described in “Materials and Methods.” SD was 5–14%.

shown to be determined by the overall characteristics of minor groove width, and resistance to digestion has been associated with minor groove narrowing (22). The structural requirements for optimal bisimidazoacridone binding to DNA have been discussed previously (12). It is therefore likely that WMC26 may alter cellular DNA structure in cells in a way that enhances recognition of those DNA regions by enzymes that are sensitive to DNA conformational changes, notably those involved in excision repair processes. A similar effect has been observed in cells treated with ditercalinium, a bifunctional intercalator which is toxic to E. coli through a process of futile repair of DNA regions bound noncovalently by the compound (23, 24).

Our experiments with UVr-deficient E. coli strains showed that WMC26 is more toxic to the repair-competent wild-type bacteria than to repair-deficient strains, and suggest that its mechanism of toxicity to prokaryotes may be comparable to that of ditercalinium.

The toxicity of ditercalinium in eukaryotic cells has been extensively studied (25, 26) and is thought to stem from the degradation of mitochondrial DNA through a similar process. Ditercalinium-treated cells show accumulation of compound in mitochondria, which are known to preferentially accumulate cationic, hydrophobic species. This process is irreversible and slow, and ultrastructural studies (21, 27) have shown mitochondrial swelling and degradation within several days of treatment. Within this time, cellular ATP and pyrimidine pools slowly decline due to a decrease in synthesis of mitochondrial membrane enzymes. Our results show that WMC26 is 1000-fold more toxic to colon carcinoma cells with competent UV repair capacity than to xeroderma pigmentosum cells, which are defective in their ability to repair UV damage to DNA, or melanoma cells, which often exhibit a high frequency of mutations characteristic of UV carcinogenesis usually attributed to a repair deficiency (28, 29). Ditercalinium was also more toxic (10-fold) to the colon carcinoma cells than to melanoma, but only at μM concentrations, and was overall 100-fold less toxic than WMC26 in vitro (12). It therefore appears that in eukaryotic cells as well, recognition of altered DNA structure by repair enzymes has a role in the enhanced toxicity of WMC26, although this may not be the sole source of this compound’s extremely high toxicity. Our previous work has indicated that WMC26 may bind to DNA by other modes beside bisintercalation (12); however, it is known that incision of undamaged DNA by excision nucleases can be promoted by noncovalent DNA-binding compounds other than bisintercalators (30).

Our cell cycle analysis of colon carcinoma and melanoma cells during treatment with μM concentrations of WMC26 and ditercalinium show that within the first 2 days after exposure, both drugs induced a profound suppression of the S-phase in the sensitive colon tumor cells. While ditercalinium appeared to only produce a G1-G0 arrest, as previously reported (25), WMC26 also blocked G2-M cell cycle progression, perhaps a sign that it inhibits growth of a broader population of asynchronously growing cells by affecting more than one aspect of cell function. Again, this could be another reason for its higher toxicity. We can speculate that the inhibition of DNA synthesis by both drugs is a result of the recognition of the noncovalently DNA-bound drugs by excision repair enzymes, and that enhanced, unsuccessful repair processes are responsible for DNA degradation. Treatment of the more resistant melanoma cells with both drugs induced no arrest of growth, suggesting that μM drug concentrations are not sufficient to trigger that process in the repair-deficient cells within that period of time.

Our confocal microscopy studies showed further similarities between the cellular distribution of WMC26 and ditercalinium. The fluorescence of WMC26 is known to be enhanced 100-fold upon binding to DNA (data not shown). We observed very intense fluoresent.

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N. Scudiero, personal communication.
cence in the cytoplasmic perinuclear area of both HCT116 and SK-MEL2 cells as early as 1 h after exposure to the compound, suggesting efficient uptake of the drugs into cytoplasmic organelles. The distribution pattern of fluorescence was similar between WMC26 and ditercalinium (data not shown), a known mitochondrial binder, while the latter produced a much less intense fluorescence. All cells showed this intense compound fluorescence after WMC26 treatment in spite of repeated washing of the cultures, indicating that the drug was firmly bound within cytoplasmic organelles. Detectable earlier in HCT116 and later in SK-MEL2 cells was an extensive vacuolization of the fluorescent regions, which suggested a degradative process, while cell nuclei showed no detectable fluorescence nor morphological alterations even after prolonged treatment. It is also significant that our confocal microscopy analysis of colon carcinoma xenografts from WMC26-treated animals showed intense drug fluorescence and extensive cytoplasmic vacuolization, strongly suggesting that the same mechanism of cellular toxicity occurs in vivo in this system.

Our studies indicate that the new bisimidazoacridone, WMC26, is a potent, selective growth inhibitor of colon carcinoma cells in vitro and in vivo. One mechanism for this effect appears to be the sensitive recognition by cellular excinucleases of mitochondrial DNA distortions produced by the strong, noncovalent binding of the drug, although interaction of WMC26 with other cytoplasmic components cannot be ruled out by these studies. Our experiments indicate that this process ensues a slow, irreversible growth arrest in sensitive cells and a subsequent process of repair-induced mitochondrial DNA degradation. Due to the apparent efficiency of this process in human colon carcinoma cells compared to repair-deficient cells, much lower drug concentrations are sufficient to induce growth arrest and cell death in these tissues. As with many other toxic drugs and some carcinogens, the structure of WMC26 appears to favor its uptake, entrapment, and transport via membranous cytoplasmic organelles, rather than directly to the nuclei of cells (31, 32). The ratio of membrane to DNA in mitochondria is 1,000 to 10,000 times greater than in nuclei, therefore a relatively high mitochondrial uptake is consistent with the high lipophilic character of the molecule. Its lipophilicity may also account for its resistance to MDR (33) as indicated by its equal toxicity to adriamycin-sensitive and resistant cell lines (data not shown). Furthermore, this may be responsible for appropriate drug distribution in vivo and contribute to its selective toxicity to HCT116 xenografts, although underlying differences between excision repair in rodent and human cells cannot be excluded (34).

The similarities between structures and antimutator activities of ditercalinium and WMC26 are apparent. Although the former is known to bisintercalate mitochondrial DNA and induce cellular toxicity in a unique way involving excision repair, the precise details of this mechanism are yet unclear. Mammalian cell mitochondria have recently been shown to have no detectable nucleotide excision repair capacity (35), while being capable of base excision repair (36, 37). The monointercalator daunomycin has been shown to selectively react with mitochondrial DNA, blocking its transcription, and its antimutator effect is thought to result from a blockage of mitochondrial biogenesis (34). The interaction between transcription and repair process has been shown in many systems (38, 39), and excision repair-deficient tissues have been observed to also be deficient in RNA synthesis following UV damage (40). WMC26 has high affinity for DNA, localizes in the cytoplasm, and our previous studies have shown that bisintercalation is not the only possible mode of binding for this compound, therefore its high activity could be the result of multiple effects on mitochondria and other cellular organelles. It is of interest to note that the current drug of choice for treating colon tumors is 5-fluorouracil, which is also thought to derive its toxicity from a futile base excision repair process resulting from sharp alterations in cellular pyrimidine pools (41). Studies of ditercalinium have suggested that degradation of mitochondrial DNA directly impairs the coding and synthesis of mitochondrial enzymes responsible for ATP production and pyrimidine synthesis (42). While ATP deficiencies can be tolerated for a period of time by concomitant stimulation of glycolysis, a more critical condition is the lowering of pyrimidine pools, which has recently been suggested to be the underlying mechanism of cell death by ditercalinium. We have shown that bisimidazoacridones can be more toxic and selective than ditercalinium, since WMC26 appears to target colon tumor cells due to their particular requirements for recognition of DNA damage and probably other as yet undefined mechanisms. The lack of systemic toxicity shown by WMC26 in our in vivo studies suggest that these compounds have excellent potential as clinically useful antimutator drugs.

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