Mode of Action of a New Indolocarbazole Anticancer Agent, J-107088, Targeting Topoisomerase I

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text

ABSTRACT

J-107088 6-N-(1-hydroxymethyl-2-hydroxy)ethylamino-12,13-dihydro-2,10-dihydroxy-13-(β-d-glucopyranosyl)-5H-indolo[2,3-a]pyrrolo[3,4-c]carbazole-5,7(6H)-dione is a new derivative of NB-506, an indolocarbazole antitumor agent. J-107088 induced single-strand DNA cleavage only in the presence of topoisomerase I (top1) more effectively than NB-506 or camptothecin. The preferable sequences of the DNA cleaved by J-107088 were C/T ↓ G as in the case of NB-506. This base-preference of J-107088 in top1-mediated cleavage was different from that of camptothecin, which was T ↓ G/A. Top1 poisons stabilize the complex between DNA and top1 (cleavable complex). This cleavable complex is released on addition of a high concentration of monovalent cation or removal of top1 poisons. The complex induced by J-107088 was quite stable; it was scarcely released on the addition of NaCl or dilution of J-107088, contrary to the case with camptothecin and NB-506. J-107088-inducing complexes were also stable in cultured cells, when the compound was added to the culture medium. These unique activities of J-107088 on top1 that differed from those of camptothecin and NB-506 may be relevant to its more potent in vitro antitumor efficacy in a human tumor xenografted nude mouse model.

INTRODUCTION

DNA top12 is a very important anticancer target. The top1 poisons irinotecan and topotecan have been used for the treatment of several types of human cancers, and 9-aminocamptothecin and 9-nitrocamptothecin are now in Phase II/III tests. In addition to these camptothecin analogues, new indolocarbazole compounds have been reported as top1 poisons. Among them, NB-506 is one of the most potent top1 poisons showing significant antitumor activity in vitro and in vivo (1, 2). Whereas camptothecin and its analogues selectively enhance DNA cleavage at a T ↓ G/A site, NB-506 enhances cleavage at a C/T ↓ G site (3). Because NB-506 shows a very wide chemotherapeutic spectrum against both murine tumors and human tumor xenografts in mice (2), Phase I studies were conducted on it at the National Cancer Center Hospital East, Chiba, Japan in 1993–1995. During the course of clinical trials of NB-506, we continued to synthesize derivatives of NB-506, to obtain more potent compounds. Among several hundred compounds thus far synthesized, J-107088 turned out to be superior to NB-506 for targeting top1. J-107088 contains an alcohol group instead of the formyl group in NB-506 at position N-6 and two hydroxyl substitutions at positions 2 and 10, which turned out to be superior to NB-506 for targeting top1. J-107088 among several hundred compounds thus far synthesized, J-107088 was C/T ↓ G as in the case of NB-506. This base-preference of J-107088 in top1-mediated cleavage was different from that of camptothecin, which was T ↓ G/A. Top1 poisons stabilize the complex between DNA and top1 (cleavable complex). This cleavable complex is released on addition of a high concentration of monovalent cation or removal of top1 poisons. The complex induced by J-107088 was quite stable; it was scarcely released on the addition of NaCl or dilution of J-107088, contrary to the case with camptothecin and NB-506. J-107088-inducing complexes were also stable in cultured cells, when the compound was added to the culture medium. These unique in vitro activities of J-107088 on top1 that differed from those of camptothecin and NB-506 may be relevant to its more potent in vitro antitumor efficacy in a human tumor xenografted nude mouse model.

MATERIALS AND METHODS

Cells and Enzymes. PSN-1 and HCT116 cells were gifts from Dr. M. Terada and Dr. N. Shindo-Okaeda, respectively, of the National Cancer Center Research Institute (Tokyo, Japan). MCF-7 and their multidrug resistant cells MCF-7 AdR were provided from National Cancer Institute (Bethesda, MD). LX-1 cells were provided by Dr. K. Komiyama of Kitasato Institute (Tokyo, Japan). MKN-45 and PC-13 cells were purchased from Immuno Biological Laboratories (Gunma, Japan). Other cell lines were purchased from the American Type Culture Collection (Rockville, MD). All of the cells were cultured in DMEM or RPMI 1640 supplemented with 10% fetal bovine serum. Recombinant mouse top1 containing six histidine residues at the NH2-terminal was produced in Spodoptera frugiperda (Sf9) cells using a baculovirus expression system. Histidine-fused Top1 was purified from the cell extract on a hydroxyapatite column and a Ni-NTA column (QIAGEN; Hilden, Germany; Ref. 3). One unit of these enzymes was defined as the activity that completely released 0.4 μg of supercoiled pBR322 DNA.

J-107088 and Other Anticancer Drugs. J-107088 (Fig. 1) is a new indolocarbazole antitumor agent. J-107088 was C/T ↓ G as in the case of NB-506. This base-preference of J-107088 in top1-mediated cleavage was different from that of camptothecin, which was T ↓ G/A. Top1 poisons stabilize the complex between DNA and top1 (cleavable complex). This cleavable complex is released on addition of a high concentration of monovalent cation or removal of top1 poisons. The complex induced by J-107088 was quite stable; it was scarcely released on the addition of NaCl or dilution of J-107088, contrary to the case with camptothecin and NB-506. J-107088-inducing complexes were also stable in cultured cells, when the compound was added to the culture medium. These unique in vitro activities of J-107088 on top1 that differed from those of camptothecin and NB-506 may be relevant to its more potent in vitro antitumor efficacy in a human tumor xenografted nude mouse model.
medium for 5, 15, 30, 60, and 120 min. After incubation, the cells were lysed with 1.5% SDS-5 mM EDTA solution and subjected to KCl/SDS precipitation assay as described previously (7).

Antitumor Spectrum In Vitro. Samples of $1 \times 10^6$ cells were plated in 96-well plates 1 day before the start of the assay. Then, sequentially diluted test compounds were added to each well, and incubation was continued for 72 h. Growth of cells was estimated by a sulforhodamine B dye-staining method (8).

RESULTS

Induction of Top1-mediated DNA cleavage. J-107088 induced single-strand cleavage in pBR322 plasmid DNA only in the presence of top1. The EC$_{50}$ values of J-107088, NB-506, and camptothecin were 0.05, 0.27, and 0.42 μM, respectively (Fig. 2). As reported about many indolocarbazoles including NB-506 (1, 9), J-107088 did not induce double-strand DNA cleavage in the presence of topoisomerase II (data not shown). And J-107088 does not belong to the staurosporine family of kinase inhibitors (data not shown). Therefore, the mechanism by which J-107088 inhibited top1 was similar to, but more potent than, those of NB-506 and camptothecin.

DNA, cleaved by incubation with top1 poisons, was found to be religated by increasing concentrations of NaCl or decreasing concentrations of top1 poisons in an in vitro system. When 0.35 mM NaCl was added to the DNA cleavage reaction, camptothecin-induced DNA nicking rapidly decreased to almost completely religated within 5 min (Fig. 3A). In contrast, DNA cleavage induced by J-107088 remained almost unchanged even 15 min after the addition of NaCl. The cleavable complex formed with NB-506 showed intermediate stability between those with camptothecin and those with J-107088. When the concentration of top1 poisons was decreased by dilution, the cleavable complex dissociated and, consequently, the level of nicked DNA was reduced (Fig. 3B). The speed of release of the J-107088-induced cleavable complex was much slower than that of NB-506 or camptothecin. These results suggested that the cleavable complex formed by J-107088 tended to be more stable than that formed by NB-506 or camptothecin.

Nucleotide Sequence of Preferable Cleavage Sites. The cleavage sites that are preferentially enhanced by J-107088 were analyzed using a 3' end-labeled 258-bp DNA fragment. J-107088 did not enhance DNA cleavage in the absence of top1 (Fig. 4, Lane 14). There were some common sites at which J-107088, NB-506, and camptothecin acted.
Thecin enhanced DNA cleavage (Fig. 4, open arrowhead). Most of these common sites had T and G at the \(-1\) and \(+1\) position, respectively, of the cleavage sites. In addition to these common sites, J-107088 and NB-506 enhanced the cleavage at several specific sites (Fig. 4, closed arrowhead). These J-107088 and NB-506-specific sites had C and G at the \(-1\) and \(+1\) position, respectively, of the cleavage sites. The pattern of cleavage by J-107088 was almost the same as that by NB-506. There were no other apparent rules of base preference of cleavage sites except for the bases at the \(-1\) and \(+1\) positions.

**DNA-Protein Complex Formation in Cells.** J-107088 increased formation of the DNA-protein complex in a time-dependent manner for up to 3 h. When J-107088 was removed from the medium, the amount of protein-linked DNA in cells decreased very slowly, and two-thirds of the complex still remained 2 h after drug removal (Fig. 5A). In contrast, the DNA-protein complex formed by camptothecin increased immediately on addition of the drug and rapidly decreased on withdrawal of the drug (Fig. 5C). Results with NB-506 were intermediate between those with J-107088 and camptothecin; the complex increased gradually and was saturated 1 h after exposure to the drug. The complex in the cells was gradually decreased after withdrawal of NB-506, disappearing almost completely after 2 h (Fig. 5B). Thus, the DNA-protein complex induced by J-107088 persisted for longer after removal of the drug from the culture medium than those formed by NB-506 and camptothecin.

**Antitumor Spectrum of J-107088 on Various Human Cancers in Vitro.** The cytotoxic activities of J-107088 on various human cancer cells were measured by a 72-h continuous exposure assay, and the results were compared with those for NB-506, camptothecin, Adriamycin, etoposide, and cisplatin (Table 1). The IC_{50} values of...
J-107088 on the 11 cell lines ranged from 0.0015 to 0.23 μM. The antitumor spectrum of J-107088 was similar to that of NB-506 but far higher against all of these cell lines. The spectrum of J-107088 was quite different from those of the other anticancer agents tested. MCF-7 AdrR were 40- and 200-fold more resistant than parental MCF-7 cells to Adriamycin and Taxol, respectively. In contrast, MCF-7 AdrR cells showed only 1.6-fold and 2.7-fold greater resistance than the parental cells to J-107088 and NB-506, respectively (Table 2). Thus, we concluded that J-107088 was effective against cancer cells that acquired resistance to Adriamycin or Taxol because of overexpression of p-glycoprotein (gp-170).

**DISCUSSION**

J-107088 was categorized as a top1 poison that is a cleavable complex-forming inhibitor because it inhibited top1 through stabilization of the DNA-enzyme complex and enhanced single-strand DNA cleavage. In this characteristic, J-107088 resembled camptothecins. However, the potency, the stability of the cleavable complex, and the preferable sequence of cleavage sites of J-107088 were quite different from those of camptothecins.

Enhancement of topoisomerase-mediated DNA cleavage is likely to be caused by the following two mechanisms; acceleration of the cleavage process and suppression of the resealing process (9, 10). Because the addition of NaCl inhibits the cleavage reaction by blocking the association of top1 with DNA, the effect on the resealing process can be highlighted at a high salt concentration (11). J-107088-induced DNA cleavage was not resealed on addition of NaCl. This result suggested that the inhibitory effect of J-107088 on the resealing process was quite potent. It is also known that restoration of cleavage is caused by the removal of top1 inhibitors. When the concentration of J-107088 was decreased by diluting the reaction mixture, the decreasing speed of nicked DNA was obviously slower than those of NB-506 and camptothecin. These results demonstrated that the cleavable complexes formed by J-107088 were more stable than those of complexes with NB-506 and camptothecin.

In analyses using cultured cells, J-107088 increased the DNA-protein complexes time-dependently during incubation, and considerable amounts of the complexes remained after withdrawal of J-107088. In contrast, the DNA-protein complex formed by camptothecin rapidly disappeared after removal of the drug. The rate of formation of J-107088-induced cleavable complex in cells was much slower than that of camptothecin. The level of DNA-protein complex was higher with camptothecin as compared with J-107088 for short incubation times (e.g., < 30 min.), although J-107088 showed higher potency to purified top1 than camptothecin. The reason for this discrepancy is not clear. The amount of complex was probably determined by the concentrations of the drug in the cells and the potency of the top1 poisons. There are at least two possibilities. One is that the uptake of J-107088 to cells may be very much slower than camptothecin. Another possibility is that J-107088 inhibits the formation of cleavable complex because J-107088 intercalates to DNA (data not shown). The amount of complex remaining after the removal of drugs was probably determined by the concentrations of the drug remaining in the cells and the stability of the cleavable complexes. Although, the cellular concentration of J-107088 was not investigated, the high stability of the J-107088-induced cleavable complex possibly contributed to the duration of stability of its intracellular complex.

J-107088 showed a very different antitumor spectrum in vitro pattern from those of camptothecin, Adriamycin, etoposide, and cisplatin. J-107088 was also effective against multidrug resistance cell lines which overexpress gp170. Previously, we demonstrated that the accumulation of J-107088 may play crucial roles in its activity and selectivity against various tumors as in the case of NB-506.

J-107088 differs from NB-506 by two substitutions (Fig. 1). Although both modifications are necessary to increase the top1 inhibition and cytotoxicity, the shift of OH groups is likely to be more critical. Similar to the result reported by Zembower et al. (12), the shift of OH groups of NB-506 from 1,11 to 2,10 positions considerably increased the potency of top1 inhibition and cytotoxicity. Furthermore, Bailly et al. in collaboration with us showed that the position of OH groups of NB-506-type drugs affected the DNA binding activities and potency of top1 inhibition (13). The shift of OH groups from 1,11 to 2,10 without the change of substituent at the 6-N position of NB-506 abolished the DNA binding activity and changed its preferable sequence of DNA cleavage from C/T ↓ G to T ↓ G. J-107088 was bound to DNA (data not shown), and its preferable sequence of DNA cleavage was C/T ↓ G (Fig. 4). The replacement of NHCHO of NB-506 to NHCH(CH3)OH2 without the change of

<table>
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<tr>
<th>Cell line</th>
<th>J-107088*</th>
<th>NB-506</th>
<th>Camptothecin</th>
<th>Adriamycin</th>
<th>Etoposide</th>
<th>Cisplatin</th>
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<tr>
<td>UM-UC-3</td>
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<td>0.056</td>
<td>0.17</td>
<td>7.3</td>
<td>1.9</td>
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* ( ), relative resistance.
position of OH groups increased the potency of top1 inhibition, DNA binding, and cytotoxicity (14, 15). Thus, the potency of top1 inhibition, DNA binding activity, and cytotoxicity of NB-506-type drug may be determined by the combination of the position of OH groups and substituent at the 6-’position.

All of these in vitro data suggest that J-107088 is a new type of top1 inhibitor with different and superior characters to other anticancer drugs currently used clinically. These finding are supported by the superior in vivo efficacy of J-107088 in a human tumor xenographic nude mouse model (16).

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