Drugs of the Future 1999, 24(12): 1311-1323

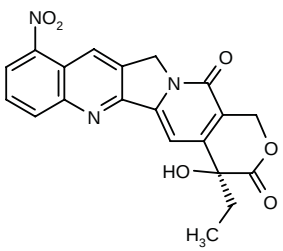
CCC: 0377-8282/99

9-Nitrocamptothecin

Antineoplastic
Antiviral
DNA Topoisomerase I Inhibitor

Rubitecan (USAN)
RFS-2000
9-NC
Camptogen™

9-Nitro-20(*S*)-camptothecin
4(*S*)-Ethyl-4-hydroxy-10-nitro-1*H*-pyrano[3',4':6,7]indolizino[1,2-*b*]quinoline-3,14(4*H*,12*H*)-dione



$C_{20}H_{15}N_3O_6$ Mol wt: 393.3590

CAS: 091421-42-0

EN: 241383

Introduction

9-Nitrocamptothecin (9-NC) is a semisynthetic derivative of the indole alkaloid, camptothecin (CPT), produced by several plants that grow in Asia, including *Camptotheca acuminata*, *Mappia foetida*, *Merriliodendron megacarpum* and *Ervatania heyneana*. The preparation of 9-NC has been described (1). Briefly, purified CPT is dissolved/suspended in concentrated H_2SO_4 and, while continually stirred, is nitrated. Eventually, a crude mixture of 9-NC and 12-NC is obtained, which can be loaded on a column of silica gel to first elute 9-NC. A subsequent reduction of 9-NC yields 9-aminocamptothecin (9-AC). The chemical structures of CPT, 9-NC and 9-AC are shown in Figure 1A. The final, purified 9-NC, 12-NC and 9-AC products are yellow and yellow-orange powders, respectively, and, like CPT, are insoluble in water. When mice with L1210 leukemia were treated with these compounds, 9-NC and 9-AC exhibited a markedly higher antitumor activity than CPT, whereas 12-NC was inactive (2). It was suggested at that time that 9-NC may be a prodrug for 9-AC because of enzymatic hydrolysis or reduction despite drastic electronic differences (1). The structure of various CPT derivatives determines their activity (3, 4) and the carboxylate (*i.e.*, open E-ring) form has much less antitumor activity than the lactone (*i.e.*, closed E-ring) form of CPT as demonstrated in studies with animals and cultured cells (Fig. 1B) (3, 5). 9-NC and 9-AC exhibit higher inhibition of cell proliferation and antitumor activities and much less toxicity than CPT and result in complete regression of various human tumors xenografted in immunodeficient athymic (nude) mice, while tumor regrowth is observed more frequently following complete tumor regression in animals treated with CPT (6-11).

9-NC is chemically more stable than 9-AC, which is readily oxidized during handling and use, and generates degradation products that are toxic to normal cultured cells and mice. Since 9-NC is the precursor of 9-AC, it can be prepared at a much higher yield and considerably lower cost. In pharmacokinetic studies, a 9-NC metabolite was identified by mass spectroscopy to be 9-AC in the plasma of a human, dog and mouse that orally received 9-NC and in the media of cells exposed to 9-NC *in vitro* (12, 13). These findings indicated that the cells can metabolically convert 9-NC to 9-AC. Further studies with a large variety of human malignant cell lines and normal cells that propagate for a limited time in culture have led to the following conclusions: regardless of tissue origin, all cell types tested are capable of converting 9-NC to 9-AC; cells that acquired resistance to 9-NC retain the ability to convert 9-NC to 9-AC; 9-NC-lactone, but not

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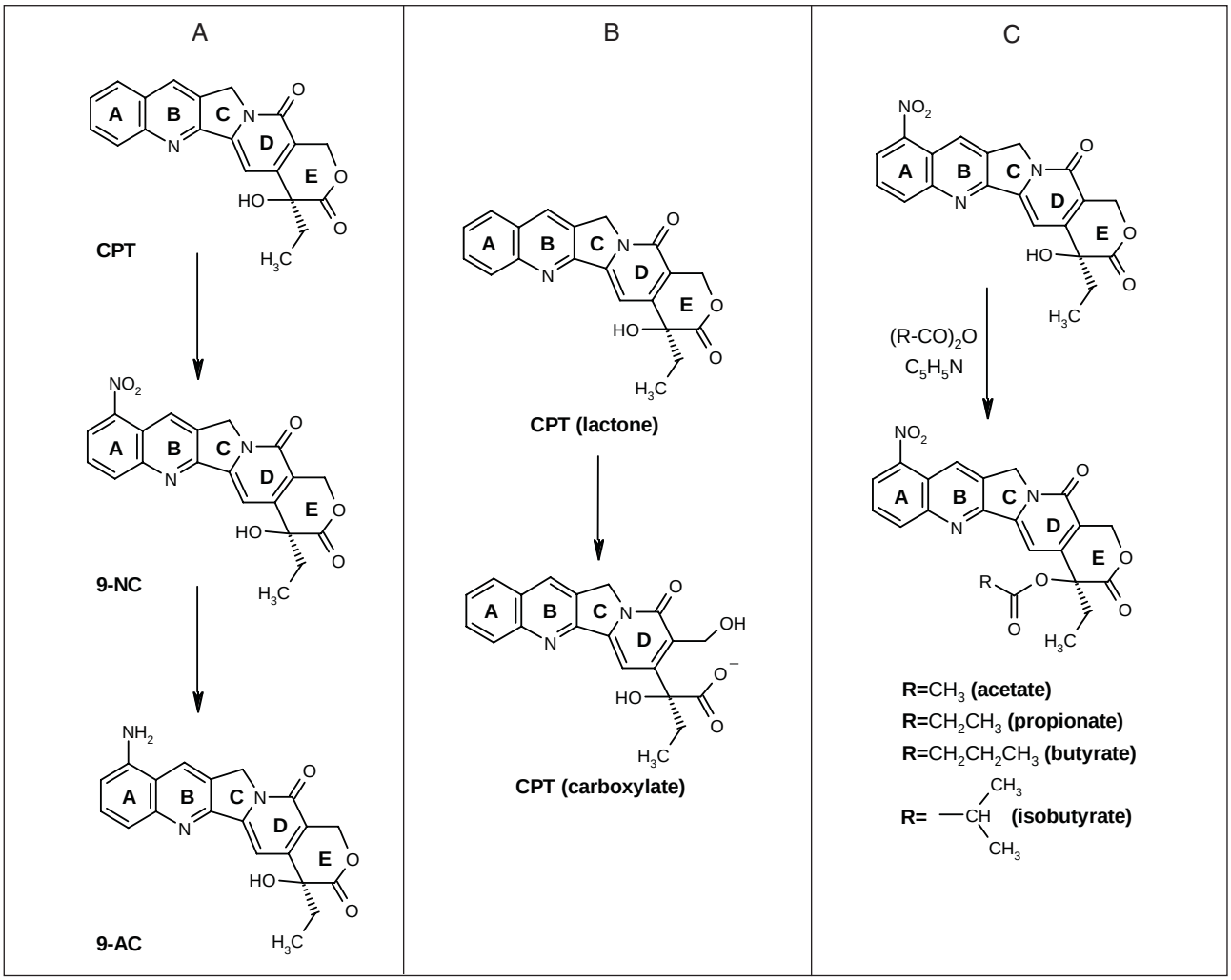

Fig. 1. Chemical structures of CPT, 9-NC, 9-AC and 9-NC esters. A) Precursor, camptothecin (CPT), and the metabolic product, 9-aminocamptothecin (9-AC), of 9-NC. B) Lactone (*i.e.*, closed E-ring) and carboxylate (*i.e.*, open E-ring) forms of CPT. C) Esterification process of 9-NC and generation of 9-NC esters at position 20 of the E-ring.

9-NC-carboxylate can be converted to the corresponding 9-AC form in presence of human serum albumin, apparently because a cellular reductase(s) recognizes the lactone but not the carboxylate form; and maximal conversion of 9-NC to 9-AC takes place at a media pH around 6 (14). These observations were of apparent clinical and economic importance and will be discussed below.

Interference of 9-NC with Topoisomerase I

Like all biologically active CPT congeners, 9-NC interferes with the mechanism of action of topoisomerase I (Top1), a nuclear enzyme present in all mammalian cells (15).

Top1 and another enzyme, topoisomerase II (Top2), are nuclear enzymes that can modulate the topological state of chromatin DNA by introducing transient DNA nicks and allowing the removal of excessive supercoils. Therefore, Top1 and Top2 modulate several vital molecular processes, including DNA replication and recombination, RNA transcription and chromosomal decondensation. In general, Top1 induces breaks on one strand, whereas Top2 induces breaks on both strands of supercoiled DNA, resulting in the formation of covalent complexes of Top-DNA fragments, termed “cleavable complexes”. This allows for removal of supercoils followed by an enzymatic DNA ligation (16-18). The biologically active CPT analog binds to the cleavable complex subsequent to the DNA cleavage step and inhibits the ligation process. Formation of the Top1-DNA-CPT complex has been demonstrated both in a cell-free system and in cultured mammalian cells (15, 19, 20). The CPT analog can bind to the Top1-DNA complex, but not to isolated Top1 or purified DNA (15). Structure-activity studies have determined that the CPT lactone has to be in the

S-configuration in order to bind to the cleavable complex (21). CPT preferentially stabilizes Top1-mediated cleavage of T-G linkages (22, 23), following formation of the covalent bond between a tyrosine residue of the enzyme and the 3'-phosphate group of thymidine (24), and interacts specifically and intimately with guanine residues of the DNA-Top1 complex (25). The exact CPT-binding site remains unknown. Prolonged CPT-induced stabilization of the Top1-DNA complex results in conversion of the single-strand breaks to irreversible double-strand breaks (25) followed by a characteristic internucleosomal degradation of chromatin DNA, that ultimately leads to cell death. CPT effectively inhibits the breakage-ligation mechanism when the target cell is in the replicative phase, *i.e.*, S phase of the cell cycle, and contains active Top1 (26-28). In addition, several other factors and/or events may regulate the CPT-induced cytotoxicity including the rate of DNA repair process, proteins that regulate or execute cell death, *etc*.

Preclinical Studies of 9-NC as a Single Agent

In the presence of 9-NC certain leukemia cells die by apoptosis when progressing through the S phase of the cell cycle, while other leukemia cells treated with 9-NC accumulate in the G_2 phase and resist death (29). Also in the presence of 9-NC, transformed human breast, ovarian, prostate, lung and malignant melanoma cells that do not form tumors when xenografted in nude mice, accumulate at the S/G_2 boundary of the cell cycle and remain there for a prolonged period of time. In contrast, cells that form tumors in nude mice are arrested in the S phase in the presence of 9-NC and subsequently die by apoptosis (11, 12, 30).

Our studies have generated one important conclusion from a clinical viewpoint: lower 9-NC concentrations applied for long periods of treatment are more effective than higher concentrations applied for short periods of treatment in inducing apoptosis (13). It is also of interest that 9-NC treatment of certain nontumorigenic cells may result in a fraction of nondividing hyperdiploid cells (11, 13), in agreement with observations reported on various leukemia cell types treated with CPT (31). It is not yet understood why 9-NC is cytostatic for nontumorigenic and cytotoxic for tumorigenic cells *in vitro*. One possibility is that cell cycle controls correlate with this differential response of the cells to 9-NC. For example, lack of arrest in G_1 or G_2 phase of the cell cycle may not provide the cell with the time required for DNA repair and may result in damage overload that cannot be repaired. In this regard, it has been shown that CPT-induced arrest in G_2 involves the protein kinase cdc2/cyclin B (32), which is required for the G_2-M phase transition. Like other agents that damage DNA and result in apoptosis, CPT induces a transient and unscheduled activation of cdc2/cyclin B during the period that follows DNA damage but precedes apoptosis (33). Hence, deregulation of cyclins, cell cycle-regulated kinases and phosphatases, p53 mutations, expression of specific genes such as *c-jun, cdc2, c-myc*, p21/WAF1 and Bcl-2 family proteins may correlate with drug-induced cytostasis or cytotoxicity (34-38). Nevertheless, this selective cytotoxicity of 9-NC, and perhaps other CPT congeners, against tumorigenic cells *in vitro* and *in vivo* is a property of this drug not observed in other known anticancer drugs. It appears, however, that although the presence of Top1 is required, it is not sufficient for induction of cytotoxicity by CPT congeners in cycling cells (39, 40).

Preclinical Studies of 9-NC Combination Treatments

In general, an anticancer drug is rarely used alone, but usually in combination with another agent(s) based upon a rationale developed in preclinical studies with cell culture or animal models or observations from earlier clinical studies. The combination treatments frequently applied are combination chemotherapy and combined modality. The studies summarized below include treatments with 9-NC and/or CPT, which is the less potent precursor of 9-NC.

Combination chemotherapy

Anticancer drug combinations have demonstrated much higher or improved curative efficacy, when compared to any single drug included in the combination, in animal models and cancer patients. Following generally accepted guidelines to select drugs for combination chemotherapy, these drugs must have demonstrated antitumor activity, display no cross-resistance, target different cellular processes and have nonoverlapping toxicities. Thus, sequential in vitro treatment of human colon cancer cells with CPT and etoposide yields additive cytotoxic effects provided that the two treatments are separated by more than 6 h (41). Further, we have shown that human leukemia and prostate carcinoma cells that acquire resistance to 9-NC or Top2-directed etoposide have inversely become highly sensitive to etoposide or 9-NC (42-45). These findings have supported the suggestion that a compensatory relationship exists between the two DNA-cleaving enzymes, Top1 and Top2 (46-48). We have further suggested that to maximize the effectiveness of the alternating combination chemotherapy with 9-NC the cancer cells or tumors must be treated extensively with the Top2-directed drug to ensure dependence on Top1 before 9-NC is applied. This suggestion is important from a clinical viewpoint, because a switch in dependence from one topoisomerase to the other for cell survival would allow for efficient treatment with the drug directed against the topoisomerase utilized most or exclusively by the cancer cell. Also, we have demonstrated that *in vitro* 9-NC-resistant human leukemia cells, but not etoposide-resistant cells, exhibit higher sensitivity than the parental cells when treated with vincristine (42), an anticancer drug that interferes with microtubule formation resulting in inhibition of mitosis. Collateral resistance or

insensitivity to vincristine is presumably due to the presence of high or increased levels of P-glycoprotein that inhibits both etoposide and vincristine, but not 9-NC. However, at present it is not known why increased resistance of cells to 9-NC confers increased sensitivity to vincristine. At any rate, studies of increased sensitivity to Vinca alkaloids by cells with increased resistance to 9-NC may also lead to development of a clinical protocol for an effective combination chemotherapy for specific type(s) of cancer.

Combined modality therapy

Failure of or unsatisfactory results with single modalities of therapy has led to development of combined modality therapy for the majority of cancers. Currently, combined modality therapy consists of two or more modalities, including chemotherapy, radiation therapy, surgery or hyperthermia, based on rationales derived from preclinical and early clinical investigations (49). The most promising combined modality therapies involving 9-NC are discussed below.

1) 9-NC and ionizing radiation

Studies of proliferating cultured cells treated with CPT and radiation have generated controversial results and, consequently, views of whether this combination treatment is effective (50-53). According to one view, CPT enhanced radiation-induced cytotoxicity because of alterations of the cell cycle progression by either agent and the marked S phase specificity of the lethal action of the drug (53, 54). Radiation alone preferentially kills cells at G_2-M (55). In contrast, other studies with human HeLa cells and Chinese hamster V-79 fibroblasts have shown no evidence of mutual potentiation of CPT and radiation in decreasing cell survival and altering the formation and rejoining of DNA double-strand breaks (56). Specifically, radiation and CPT applied concomitantly or in close temporal proximity were shown to interact with each other in a purely additive mode, whereas when radiation was applied 2 h or longer prior to CPT treatment, CPT-induced toxicity was apparently decreased following CPT treatment (56). These controversial results probably correlate with various parameters contributing to the complexity of CPT- and radiation-induced mechanisms and, therefore, these results should be evaluated in this context. In this regard, a recent study of irradiation combined with the metabolic product of 9-NC, 9-AC, on a mouse mammary cancer has indicated that the frequency and timing of 9-AC administration with irradiation are important factors in the design of clinical protocols for effective tumor treatment with no or low acute toxicity (57).

Experimental parameters which may result in diverse results after 9-NC/radiation therapy include cell type, drug concentrations used, duration of drug treatment, whether drug-treatment precedes or follows radiation, expression of genes that control check points of the cell cycle, *etc*. For example, although CPT and 9-NC specifically target S phase cells, the exact stage may vary within the S phase as a result of the drug concentration. In this regard, relatively high 9-NC concentrations block DNA synthesis at early S phase, *i.e.*, near G_1, whereas low drug concentrations block DNA synthesis at late S phase, *i.e.*, near G_2 (30). Therefore, radiation administered after initiation of CPT or 9-NC treatment will probably generate various results depending on the stage of S phase, *i.e.*, early, middle or late, in which the cells have accumulated at the time of irradiation. The presence of functionally active Top1 is another parameter. 9-NC added concomitantly with or immediately after radiation requires a long time for any additive or synergistic cytotoxicity to be observed because, immediately following ionizing radiation, there is a dramatic decrease in Top1 enzymatic activity (58), which apparently will result in decreased 9-NC-induced toxicity. Downregulation of Top1 activity in irradiated cells results from posttranslational modification rather than decreased synthesis of mRNA and protein (58). Also, differences in radiosensitivity may exist in cells because of loss of function of the p53 protein (59, 60) and expression of specific Bcl-2 family proteins (61-63). Finally, kinases, phosphatases and other key proteins differentially control cell cycle events in normal and cancer cells, and therefore, studies of these events in 9-NC- and ionizing radiation-induced toxicity is a prerequisite for the development of protocols of combination treatments of 9-NC with radiation, so that cancer cells will be killed while normal cells will be only minimally affected or unaffected.

2) 9-NC and hyperthermia

Application of hyperthermia combined with anticancer drugs, *i.e.*, thermochemotherapy, has been explored as a clinical strategy to enhance the therapeutic effect of several anticancer drugs, even in drug-resistant cells (64). For example, heat potentiates the cytotoxicity of cisplatinum (65) and tumor necrosis factor used to treat cultured cells and human tumors xenografted in nude mice (66-70). In related earlier studies, etoposide-induced toxicity was not enhanced by hyperthermia (70), whereas in some instances, hyperthermia protected human and rodent cells from the cytotoxic action of etoposide and amsacrine (71, 72). However, these studies cannot be compared since different experimental models (*i.e.*, cell types) were used. It has been shown that heat alone may induce G_1 arrest depending on the cell type, and this arrest correlates with increases in the level of p16 (73), a protein that acts as a negative regulator of the cell growth (74, 75).

With regard to CPT, no significant increase was reported for its cytotoxicity *in vitro* when mouse mammary tumor cells were exposed to the drug at 42 ^{o}C compared with the same drug exposure at 37 ^{o}C (65). However, combination treatment with CPT and hyperthermia killed more cells than CPT treatment alone in mice carrying FSAIIC fibrosarcoma (76). A recent study in our

laboratory has demonstrated that the sequence of treatments with hyperthermia and 9-NC is important in order to increase or decrease the cytotoxic action of 9-NC against human leukemia HL-60 cells *in vitro* (77). In fact, there is a marked similarity observed on the interacting action of 9-NC and etoposide with hyperthermia at 43 ºC for 60 min (78), in part because of the similar mechanisms of action of Top1 and Top2.

9-NC as an Agent of Cell Differentiation

The concept of "differentiation chemotherapy" is based upon observations that cytostatic drug treatment of malignant cells in culture, and in some instances *in vivo*, can induce differentiation into normal, less malignant cells (79-81). Studies of CPT inducing cell differentiation have been conducted with human hematopoietic (leukemia) cell lines. It was initially reported that CPT was a potent agent of cell differentiation for several human leukemia cell lines including HL-60, U-937, ML-1 and K-562 (82-84). Differentiation was assessed by monitoring changes in differentiation markers including ability to reduce nitro-blue tetrazolium, synthesis of α-naphthyl acetate and naphthol AS-D chloroacetate esterases, synthesis of Fc-receptors and specific globin mRNAs and ability to phagocytize. These studies showed that CPT-treated ML-1 and U-937 cells differentiate along the granulocytic lineage, whereas HL-60 and K-562 cells differentiate along the monocytic and erythrocytic lineage, respectively (82-84). In contrast, another study showed that, like CPT-treated HL-60 and THP-1 cells, CPT-treated U-937 cells express specific markers of monocytic differentiation (85). However, regardless of the reported differentiation lineage of CPT-treated U-937 cells, these cells and all other leukemia cells examined are induced to differentiate to more mature cells in the presence of CPT (82-85). Continuous exposure of U-937 cells to increasing 9-NC concentrations results in development of resistance to the drug, and this correlates well with an increase in the "normalcy" of the cells as determined by specific molecular markers and decreased ability or inability of the cells to induce tumors when xenografted in nude mice (86). Apparently, these results may have important implications in the differentiation chemotherapy of leukemia patients treated with CPT or 9-NC. It is not yet known how CPT or 9-NC induces differentiation of human leukemia cells *in vitro*, but in U-937 cells this induction appears to correlate with a transient activation of transcription of the early response genes, *c-jun* and *jun-B* (87), presence of mutated Top1 (88) or lower expression of Top1 and increased expression of Top2 (89). The c-jun and jun-B genes are members of a multigene family of transcription factors (90). On the other hand, it has been demonstrated that Top1 is required for gene transcription (91, 92). Taken together these findings suggest that inhibition of Top1 by CPT can result in both down- and upregulation of expression of specific genes (85, 87). This suggestion has been substantiated by recent findings that showed involvement of Top1 in both repression and activation of gene transcription *in vitro* (92).

It has also been observed that CPT-induced leukemia cell differentiation is accompanied by inhibition of Top1 activity (82-84), increased cellular and nuclear size (85, 87) and generation of DNA strand breaks (82-85, 87). Although these events occur during the same or overlapping periods, it is likely that they are results independent and unrelated to CPT-induced mechanisms (84, 87). It has been suggested that the differentiation effect of CPT on gene expression is dependent on drug concentration, that is, low (under 20 nM) CPT concentrations induce DNA hypomethylation (93), a process that has been associated with transcriptional activation in animal cells (94, 95). Therefore, the extent of drug-induced inhibition of DNA methylation may correlate with inhibition of Top1 activity and, in turn, altered gene expression and induction of differentiation (93). Another suggestion is that CPT and other Top1 inhibitors induce cell differentiation at concentrations of 10- to 100-fold below the lethal dose, which is below the drug concentration that stabilizes the cleavable complex (96). Also, low CPT concentrations may result in unbalanced cell growth which, in turn, may result in hyperdiploidy and increased nuclear and/or cell size.

In addition to CPT, 9-NC has also been used *in vitro* in differentiation induction studies of human cancer cells from solid tissues. Thus, presence of low (*i.e.*, 20-30 nM) 9-NC concentrations to cultures of human hepatoblastoma HepG2 cells results in their arrest in the G_2 phase of the cell cycle and results in the appearance of morphological and biochemical features associated with more differentiated cells (37). Also, the G_2 arrested cells express upregulated levels of the cyclin-dependent kinase inhibitor, p21 (37), that has been implicated in induction of differentiation of cells of diverse tissue origin including melanoma cells (97, 98). Specifically, ectopic expression of p21 in human malignant melanoma cells elicited growth arrest at the G_1 phase, differentiation and suppression of tumor growth in animals inoculated with the p21-expressing cells (98). We also have conducted studies of differentiation of human malignant melanoma cells exposed to 9-NC *in vitro* (99). We have shown that after exposure of the SB1B cell line to 50 nM 9-NC, 65-80% of the cells die by apoptosis, whereas the surviving cells are arrested at the G_2 phase and subsequently develop characteristic features of normal melanocytes which include dendritic extensions, synthesis of the pigment melanin and, importantly, loss of tumorigenic ability after being xenografed in nude mice. Further, we have shown that untreated, exponentially growing or density G_1-arrested SB1B cells express p21, whereas this protein is virtually undetected in 9-NC-treated, G_2-arrested cells (99). The reverse is observed in the expression of p16, another cell cycle negative regulator, which binds to and inactivates the cyclin D/CDK4 (or CDK6) complex, thus resulting in cell cycle arrest at G_2 (100). Our results support a previous study that restoration of p16 into melanoma cells *in vitro* resulted in the appearance of morphological features of mature melanocytes (101).

Table I: Major studies of DNA viruses in presence of CPT.

DNA virus family and member(s)	Experimental system	Function(s) or effect(s) disrupted or decreased by CPT
Adenoviridae		
Ad2, Ad5	Ad2-, Ad5-infected human (HeLa) cells	Viral DNA replication, transcription, genome packaging
Papovaviridae		
SV40	Cell-free; SV40-infected monkey kidney cells	Viral DNA replication
JVC	JVC-infected human glial cells	Viral DNA replication
Herpesviridae		
HSV-2	HSV-2-infected human fibroblasts	Viral DNA replication
EBV	EBV-infected human T cells	Viral DNA replication
CMV	CMV-infected human fibroblasts	Cytopathic manifestations
Parvoviridae		
LuIII (H1)	LuIII-infected human (HeLa) cells	Viral DNA synthesis

Taken together, these results demonstrate the feasibility of using 9-NC in differentiation therapy as a clinical procedure in manipulating melanoma.

9-NC as an Antiviral Agent

In earlier studies, CPT was used as a tool to elucidate functions of viruses and their interactions with components of infected cells. These studies led to conclusions that CPT can attenuate or completely block Top1 activity required for functions of DNA or RNA viruses regardless of whether this Top1 activity was of virus or host cell origin. Viral functions affected by CPT congeners have been studied in cell-free systems utilizing purified components and/or enzymatic activities from viruses and mammalian cells, and models of virus-infected cells and animals. DNA viruses studied in the presence of CPT include the adenoviruses (Ad) 2 and 5; the papovaviruses, simian virus 40 (SV40) and JC virus (JCV); the herpesviruses, herpes simplex virus type 2 (HSV-2), Epstein-Barr virus (EBV) and human cytomegalovirus (CMV); and the parvoviruses LuIII and H1. These studies and the major observations are summarized in Table I and have been previously described more extensively (102). Importantly, the studies with JCV- and EBV-infected cells demonstrated that the CPT-induced viral inhibitory effects occurred with no apparent inhibition of the host cell transcription, translation and viability. Studies of DNA viruses exposed to 9-NC have not yet been reported. However, 9-NC is biologically more potent, but less toxic for cells, than CPT, and therefore 9-NC may develop to a highly effective drug against DNA viruses that require Top1 activity for various functions.

Although they have been investigated in a limited number of studies, CPT and 9-NC have demonstrated a remarkable activity against murine, horse and human retroviruses. The studies were conducted in cell-free systems, virus-infected cells in cultures and virus-infected animals using the Moloney murine leukemia virus (Mo-MLV) that induces development of lymphoma in mice, the Friend murine leukemia virus (FV) that causes rapid development of erythroleukemia in mice, the equine infectious anemia virus (EIAV) that causes viremia, fever, anemia and tissue and erythrocyte destruction in horses and the human immune deficiency virus (HIV), the causative agent of AIDS. The results of these studies (103-105) are summarized in Table II.

9-NC Derivatives

The unprecedented curative ability of 9-NC against human tumors grown in nude mice was not observed to the same extent in clinical trials with cancer patients. This differential antitumor efficacy of 9-NC in the human and mouse systems can be explained by the differential bioavailability of the 9-NC lactone in these two recipients. It has been demonstrated that human serum albumin (HSA) *in vitro* exhibits 150- to 200-fold binding preference for the carboxylate relative to the CPT lactone, and that serum albumins from other species, including mouse, bind CPT carboxylate much less tightly than HSA (106-108). As a result, the equilibrium of lactone (closed E-ring) $\leftrightarrow$ carboxylate (open E-ring) favors faster carboxylate formation in humans than in mice. Wani *et al.* (109) first demonstrated that the carboxylate Na salt of CPT has only about 10% of the antitumor potency of CPT lactone in a rodent leukemia system. The correlation of antitumor activity and presence of intact lactone E-ring was further confirmed in studies of human tumor xenografts in mice treated with closed and open lactone E-ring (110).

To prolong the bioavailability of 9-NC in the plasma of patients this drug has been synthetically modified in order to delay conversion of the lactone to the carboxylate form. For this, Cao *et al.* prepared four 9-NC alkyl esters, namely 9-NC-acetate, 9-NC-propionate, 9-NC-butyrate and 9-NC-isobutyrate, by adding the appropriate prosthetic groups to position 20 of the 9-NC-lactone E-ring (Fig. 1C) (111). These esters were assayed for ability to inhibit growth and induce apoptosis in HL-60 and U-937 cells (112). Although less active than 9-NC, the

Table II: Summary of studies with retroviruses treated with CPT congeners.

Retrovirus	Model	Major findings
Mo-MLV	Mo-MLV-infected newborn mouse	CPT prevented and significantly reduced development of splenomegaly or size of enlarged spleen
FV	FV-infected adult mouse	CPT pretreatment prevented splenomegaly development after FV injection
EIAV	Chronically EIAV-infected horse cells *in vitro*	CPT inhibited viral replication with no apparent effect on proliferation and viability of host cells
HIV	HIV-infected human T cells *in vitro*	CPT inhibited virus replication and HIV infection
	Human T cells infected with latently activated HIV *in vitro*	9-NC inhibited TNF-induced activation of virus in HIV-infected cells when 9-NC treatment preceded, but not followed, TNF treatment. 9-NC induced apoptosis in latently infected, but not in uninfected cells
	Human monocytoid cells freshly infected with HIV *in vitro*	9-NC inhibited HIV replication
	Human monocytoid cells resistant to 9-NC but infected with HIV *in vitro*	9-NC failed to inhibit HIV replication

propionate and butyrate esters were still markedly active, while the other 9-NC esters exhibited little or no activity. Apparently, intact 9-NC-propionate and 9-NC-butyrate are biologically inactive and require de-esterification in order to become bioactive, *i.e.*, these esters must be converted to 9-NC by specific carboxylesterases. It is possible that 9-NC-propionate and 9-NC-butyrate are bioactivated, *i.e.*, deesterified, by HL-60 and U-937 cells, whereas the other 9-NC esters can be bioactivated by other tissue types. Tissue-dependent bioactivation of specific 9-NC esters is under investigation. However, the preliminary studies have indicated that esters and other modified forms of 9-NC can be developed as 9-NC prodrugs that can be administered at large doses with appearance of low toxicity in normal cells and tissues. At any rate, the modified 9-NC must be converted to 9-NC in order to exhibit its antitumor activity. Various modified 9-NC molecules are currently under investigation.

Clinical Studies

Clinical trials have been conducted with 9-NC orally administered in gelatin capsules. In a phase I clinical trial of 9-NC (113), 28 patients with advanced disease refractory to conventional chemotherapy received 9-NC scheduled in cycles of 5 consecutive days every week. The starting drug dose was 1 mg/m^2/day for 4 weeks with increments of 0.5 mg/m^2/day. At doses above 1.5 mg/m^2/day, WHO grade 4 toxicity occurred as anemia (8 patients; 29%), neutropenia (7 patients; 25%) and thrombocytopenia (5 patients; 18%). Grade 2 or higher nonhematologic toxicity occurred at each dose level and was manifested as nausea/vomiting (15 patients; 54%), diarrhea (9 patients; 32%), chemical systitis (7 patients; 25%), neutropenic sepsis (6 patients; 21%) and weight loss (5 patients; 18%). Alopecia was rare and appeared in patients with severe neutropenia. The dose-limiting toxicity was cystitis. Five partial responses were observed after 2-8 weeks of therapy in patients with pancreatic, breast, ovarian and hematologic tumors. Fourteen patients had a minor response or stable disease while on treatment. The MTD of oral 9-NC has been estimated at 1.5 mg/m^2/day for 5 consecutive days.

A phase II trial of 9-NC was conducted in patients with heavily refractory tubal or peritoneal cancer (114). The median number of previous chemotherapy regimens was over 3. The starting 9-NC dose was 1.5 mg/m^2/day and was administered for 4 consecutive days every week, followed by a dose elevation of 0.25 mg/m^2/day in patients with no significant side effects. Toxicity was evaluated in 31 patients. WHO grade 3 or 4 hematologic toxicity consisted of anemia (10 patients; 32%), neutropenia (8 patients; 26%) and thrombocytopenia (5 patients; 10%). Grade 2 or higher nonhematologic toxicity consisted of nausea and vomiting (26 patients; 84%), diarrhea (12 patients; 39%), weight loss (7 patients; 22%), chemical cystitis (6 patients; 19%) and neutrophilic sepsis (6 patients; 19%). 9-NC was tolerated for sustained periods of time up to 47 weeks in some patients.

In a recent 9-NC clinical study in patients with advanced pancreatic cancer (115), the median survival was 8.7 months for the 60 patients qualified for evaluation. Of these patients, 31.7% were responders (median survival 18.6 months; range 6.5-44.7+ months), 31.7% were stable (median survival 9.7 months) and 33.6% were nonresponders (median survival 6.8 months). Previously untreated and treated patients had median survival periods of 7.3 and 4.7 months, respectively.

Conclusions and Perspectives

Unquestionably, the CPT derivative 9-NC has exhibited *in vivo* and *in vitro* properties and abilities that warrant

its further investigation as a potent anticancer and antiviral drug. The diverse capabilities of 9-NC to induce apoptosis, cytostasis or cell differentiation appear to depend on the "malignant stage" and/or the type of cell. At present, there is no evidence that 9-NC and other active CPT congeners act via specific cellular receptors in inducing cell death by apoptosis. But it is well established that formation of the Top1-DNA cleavable complex, and its subsequent stabilization by the CPT congener, require ongoing DNA synthesis for drug-induced apoptosis. It is not known whether physical interactions between Top1 and other cellular components are important in the cytotoxic and differentiation effects of CPT congeners. For example, Top1 physically interacts with HMG17 (116), RNA polymerase I (117), nucleolin (118), SV40 T antigen (119, 120), p53 (121, 122) and other proteins involved in replication or transcription. Further, interaction of Top1 with the SV40 T antigen helicase has been shown to modulate Top1-DNA-CPT ternary complexes, suggesting that similar interactions between Top1 and cellular helicases may mediate the cytotoxicity of CPT congeners (123). However, it remains to be investigated whether interaction between Top1 and a specific cellular protein(s) plays a role(s) in cytotoxicy, cytostasis and differentiation induced by 9-NC and other CPT congeners.

The covalently linked cleavable complex of DNA-Top1 appears to be the major target for all CPT congeners. Formation of a stable DNA-Top1-CPT complex is an event required for the apoptotic action of all biologically active CPT congeners, but beyond this similarity these compounds, including 9-NC, exhibit diverse antitumor efficacy, pharmacology, pharmacokinetics and metabolism (6, 7, 13, 14, 124). Also, the limiting toxicity in treated patients depends on the congener. For example, the major clinical problem is diarrhea with CPT and myelosuppression with 9-NC (125). For each CPT congener the important issues are the establishment of optimal scheduling, route of administration, pharmacological formulation and clinical efficacy. Specifically for 9-NC, it appears that oral administration, treatment scheduling (5 consecutive days a week followed by 2 days without treatment) and dose (starting at 1.5 mg/m^2/day) are more or less established parameters when the drug is used as a single modality. It is also evident from the clinical trials that cancer patients without previous treatment are better responders than previously treated patients.

At present there are no clinical results of combination chemotherapy or combined modality treatments with 9-NC. However, promising results have derived from studies of combination treatment of cancer cells *in vitro* with 9-NC and etoposide. Additional combinations of 9-NC with other anticancer drugs may improve even more the beneficial outcome in treated patients. Also, studies *in vitro* and *in vivo* with ionizing radiation and 9-AC, *i.e.*, the metabolic product of 9-NC, have shown that 9-AC can enhance the radiation-induced toxicity (57, 126, 127). Such results have indicated that frequency and timing of 9-AC administration and radiation treatment are important factors to be considered in the design of clinical protocols. In conclusion, combination chemotherapy and combined modality therapies appear to be promising strategies for localized or systemic treatment of cancer, and development of appropriate treatment protocols should be of high priority.

Differentiation chemotherapy with 9-NC is another promising strategy for treatment of cancer patients, particularly those with leukemias. In this strategy, 9-NC must be used at low concentrations or doses so that it will not damage the genome but rather activate specific genes associated with cell differentiation. Also important in this strategy may be the role of DNA hypomethylation, and therefore other agents that induce DNA hypomethylation could be used to "prime" the cancer cells for 9-NC-induced differentiation. This hypothesis is currently being investigated in our laboratory using various cancer cell systems *in vitro*.

Studies of the 9-NC precursor CPT with various mammalian retroviruses and our studies *in vitro* with HIV-infected human lymphocytic and monocytic cells strongly indicate the efficacy of 9-NC as an antiretroviral agent used alone or in combination with other antiretroviral agents. However, clinical trials of 9-NC with retrovirus-infected individuals remain to be conducted. In this context, 9-NC may also prove to be a potent agent against infectious DNA viruses, and efforts should be made to pursue clinical studies with individuals infected with DNA viruses.

Finally, the remarkable ability of 9-NC, at pharmacological doses, to kill cancer but not normal cells *in vitro* and *in vivo* should be investigated in order to improve the efficacy of this drug. One possibility is to synthesize 9-NC prodrugs that preserve and slowly release the drug's antitumor activity in the human blood and other tissues. These prodrugs will allow administration of increased doses, less frequent drug treatment, continuous presence of 9-NC in the cancer tissue environment and provide adequate time for the human body to remove and, therefore, prevent accumulation of by-products toxic for normal cells and tissues (which in turn will result in a decrease in or absence of side effects). Another possibility to increase 9-NC-induced antitumor activity and decrease drug-generated side effects is to sensitize the apoptotic machinery of cancer cells exposed to this drug. Identification of components/events of the 9-NC-induced apoptotic cell death is currently underway at our laboratory.

In conclusion, the properties and abilities of 9-NC exhibited in preclinical and clinical studies and its immense possibilities to become a potent anticancer and antiviral agent make it one of the most promising drugs of the future.

Sources

The Stehlin Foundation for Cancer Research, Houston, TX (US); licensed to SuperGen Inc. (US).

Acknowledgements

The authors wish to thank the Howard Hughes Medical Institute for their support through grant No. 5-29251 to Brown University.

References

1. Wani, M.C., Nicholas, A.W., Wall, M.E. *Plant antitumor agents. 23. Synthesis and antileukemia activity of camptothecin analogs.* J Med Chem 1986, 29: 2358-63.

2. Wall, M.E., Wani, M.C. *Antineoplastic agents from plants.* Annu Rev Pharmacol Toxicol 1977, 17: 117-32.

3. Jaxel, C., Kohn, K.W., Wani, M.C., Wall, M.E., Pommier, Y. *Structure-activity study of the actions of camptothecin derivatives on mammalian topoisomerase I: Evidence for a specific receptor site and a relation to antitumor activity.* Cancer Res 1989, 49: 1465-9.

4. Hsiang Y.-H., Liu, L.F., Wall, M.E., Wani, M.C., Kirshenbaum, S., Silber, R., Potmesil, M. *DNA topoisomerase I-mediated DNA cleavage and cytotoxicity of camptothecin analogs.* Cancer Res 1989, 49: 4385-9.

5. Wall, M.E., Wani, M.C. *Chemistry and antitumor activities of camptothecins.* In: DNA Topoisomerases in Cancer, M. Potmesil and K.W. Kohn (Eds.), Oxford University Press, New York, 1991, 93-102.

6. Hinz, H.R., Harris, N.J., Natelson, E.A., Giovanella, B.C. *Pharmacokinetics of the in vivo and in vitro conversion of 9-nitro-20(S)-camptothecin to 9-amino-20(S)-camptothecin in human, dog and mouse.* Cancer Res 1994, 54: 3096-100.

7. Pantazis. P., Harris, N., Mendoza, J., Giovanella, B. *Conversion of 9-nitrocamptothecin to 9-aminocamptothecin by human blood cells in vitro.* Eur J Haematol 1994, 53: 246-8.

8. Pantazis, P., Harris, N., Mendoza, J., Giovanella, B. *The role of pH and serum albumin in the metabolic conversion of 9-nitrocamptothecin to 9-aminocamptothecin by human hematopoietic and other cells.* Eur J Haematol 1995, 55: 211-3.

9. Giovanella, B.C., Stehlin, J.S., Wall, M.E., Wani, M.C., Nicholas, A.W., Liu, L.F., Silber, R., Potmesil, M. *DNA topoisomerase I-targeted chemotherapy of human colon cancer in xenografts.* Science 1989, 246: 1046-8.

10. Pantazis, P., Hinz, H.R., Mendoza, J.T., Kozielski, A.J., Williams, L.J., Stehlin, J.S., Giovanella, B.C. *Complete inhibition of growth followed by death of human malignant melanoma cells in vitro and regression of human melanoma xenografts in immunodeficient mice by camptothecins.* Cancer Res 1992, 52: 3980-7.

11. Pantazis, P., Early, J.A., Kozielski, A.J., Mendoza, J.T., Hinz, H.R., Giovanella, B.C. *Regression of human breast carcinoma tumors in immunodeficient mice treated with 9-nitrocamptothecin: Differential response of nontumorigenic and tumorigenic human breast cells in vitro.* Cancer Res 1993, 53: 1577-82.

12. Pantazis, P., Kozielski, A.J., Mendoza, J.T., Early, J.A., Hinz, H.R., Giovanella, B.C. *Camptothecin derivatives induce regression of human ovarian carcinomas grown in nude mice and distinguish between non-tumorigenic and tumorigenic cells in vitro.* Int J Cancer 1993, 53: 863-71.

13. Pantazis, P., Kozielski, A.J., Vardeman, D.M., Petry, E.R., Giovanella, B.C. *Efficacy of camptothecin congeners in the treatment of human breast carcinoma xenografts.* Oncol Res 1993, 5: 273-81.

14. Pantazis, P., Kozielski, A., Rodriguez, R., Petry, E., Wall, E.M., Wani, M.C., Giovanella, B.C. *Therapeutic efficacy of camptothecin derivatives against human malignant melanoma xenografts.* Melanoma Res 1994, 4: 5-10.

15. Hsiang, Y.-H., Hertzberg, R., Hecht, S., Liu, L.F. *Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I.* J Biol Chem 1985, 260: 14873-8.

16. Caron, P., Wang, J.C. *DNA topoisomerases as targets of therapeutics: A structural overview.* In: Molecular Biology of DNA Topoisomerases and Its Application to Chemotherapy, T. Ando, H. Ikeda and M. Oguro (Eds.), CRC Press, Boca Raton, 1993, 1-18.

17. Molecular Biology of DNA Topoisomerases and its Application to Chemotherapy, T. Ando, H. Ikeda and M. Oguro (Eds.), CRC Press, Boca Raton, 1993.

18. Topoisomerases: Biochemistry and Molecular Biology, L.F. Liu (Ed.), Advances in Pharmacology Vol. 29A, 1994.

19. Hsiang, Y.-H., Liu, L.F. *Identification of mammalian topoisomerase I as an intracellular target of the anticancer drug camptothecin.* Cancer Res 1988, 48: 1722-6.

20. Hertzberg, R., Caranfa, M., Hecht, S. *On the mechanism of topoisomerase I inhibition by camptothecin: Evidence for binding to an enzyme-DNA complex.* Biochemistry 1989, 28: 4629-38.

21. Pommier, Y., Jaxel, C., Heise, C.R., Kerrigan, D., Kohn, K.W. *Structure-activity relationship of topoisomerase I inhibition by camptothecin derivatives: Evidence for the existence of a ternary complex.* In: DNA Topoisomerases in Cancer, M. Potmesil and K.W. Kohn (Eds.), Oxford University Press, New York, 1981, 121-32.

22. Jaxel, C., Capranico, G., Kerrigan, D., Kohn, K., Pommier, Y. *Effect of local DNA sequence on topoisomerase I cleavage in the presence or absence of camptothecin.* J Biol Chem 1991, 226: 20418-23.

23. Porter, S.E., Champoux, J.J. *The basis of camptothecin enhancement of DNA breakage by eukaryotic topoisomerase I.* Nucleic Acids Res 1989, 17: 8521-32.

24. Champoux, J.J. *DNA is linked to the rat liver nicking-closing enzyme by a phosphodiester bond to tyrosine.* J Biol Chem 1981, 256: 4805-9.

25. Leteurtre, F., Fesen, M., Kohlhagen, G., Kohn, K.W., Pommier, Y. *Specific interaction of camptothecin, a topoisomerase I inhibitor, with guanine residues of DNA detected by photoactivation at 365 nm.* Biochemistry 1994, 3: 8955-62.

26. Zhang, H., D'Arpa, P., Liu, L. *A model for tumor killing by topoisomerase poisons.* Cancer Cells 1990, 2: 23-7.

27. Hsiang, Y.-H., Lihou, M.G., Liu, L.F. *Arrest of replication forks by drug-stabilized topoisomerase I-DNA cleavable complexes as a mechanism of cell killing by camptothecin.* Cancer Res 1989, 49: 5077-82.

28. Tsao, Y.-P., Russo, A., Nyamuswa, G., Silber, R., Liu, L.F. *Interaction between replication forks and topoisomerase I-DNA cleavable complexes: Studies in a cell-free SV40 DNA replication system.* Cancer Res 1993, 53: 5908-14.

29. Pantazis, P., Mendoza, J.T., Kozielski, A.J., Natelson, E.A., Giovanella, B.C. *9-Nitrocamptothecin delays growth of U-937 leukemia tumors in nude mice and is cytotoxic or cytostatic for human myelomonocytic leukemia lines in vitro.* Eur J Haematol. 1993, 50: 81-9.

30. Pantazis, P., Early, J.A., Mendoza, J.T., DeJesus, A.R., Giovanella, B.C. *Cytotoxic efficacy of 9-nitrocamptothecin in the treatment of human malignant melanoma cells in vitro.* Cancer Res 1994, 54: 771-6.

31. Del Bino, G., Skierski, J.S., Darzynkiewicz, Z. *Diverse effects of camptothecin, an inhibitor of topoisomerase I, on the cell cycle of lymphocytic (L1210, MOLT-4) and myelogenous (HL-60, KG-1) leukemia cells.* Cancer Res 1990, 50: 5746-50.

32. Tsao, Y.P., D'Arpa, P., Liu, L.F. *The involvement of active DNA synthesis in camptothecin-induced G_2 arrest: Altered regulation of p34(cdc2)/cyclin B.* Cancer Res 1992, 52: 1823-9.

33. Shimizu, T., O'Connor, P.M., Kohn, K.W., Pommier, Y. *Unscheduled activation of cyclin B1/cdc2 kinase in human promyelocytic leukemia HL60 cells undergoing apoptosis induced by DNA damage.* Cancer Res 1995, 55: 228-31.

34. Lanza, A., Tornaletti, S., Stefanini, M., Evans, H.H., Ricanati, M., Astaldi-Ricotti, J., Pedrini, A.M. *The sensitivity to DNA topoisomerase inhibitors in L5178Y lymphoma strains is not related to a primary defect of DNA topoisomerases.* Carcinogenesis 1993, 14: 1759-63.

35. Gong, J., Ardelt, B., Traganos, F., Darzynkiewicz, Z. *Unscheduled expression of cyclin B1 and cyclin E in several leukemic and solid tumor cell lines.* Cancer Res 1994, 54: 4285-8.

36. Houghton, J.A., Harwood, F.G., Houghton, P.J. *Cell cycle control processes determine cytostasis or cytotoxicity in thymineless death of colon cancer cells.* Cancer Res 1994, 54: 4967-73.

37. Khaoustov, V.I., Ozer, A., Smith, J.R., Noda, A., Mearns, M., Krishnan, B., Stagle, B.L., Yoffe, B. *Induction of senescence-derived inhibitor of DNA synthesis gene, SDII, in hepatoblastoma (HepG2) cells arrested in the G_2-phase of the cell cycle by 9-nitrocamptothecin.* Lab Invest 1995, 73: 118-27.

38. Pandey, S., Wang, E. *Cells en route to apoptosis are characterized by the upregulation of c-fos, c-myc, c-jun, cdc2 and RB phosphorylation, resembling events of early cell-cycle traverse.* J Cell Biochem 1995, 58: 135-50.

39. Goldwasser, F., Bae, I., Valenti, M., Torres, K., Pommier, Y. *Topoisomerase I-related parameters and camptothecin activity in the colon carcinoma cell lines from the National Cancer Institute anticancer screen.* Cancer Res 1995, 55: 2116-21.

40. Beidler, D.R., Chang, J.Y., Zhou, B.S., Cheng, Y.C. *Camptothecin resistance involving steps subsequently to the formation of protein-linked DNA breaks in human camptothecin-resistant KB cell lines.* Cancer Res 1996, 56: 345-53.

41. Bertrand, R., O'Connor, P.M., Kerrigan, D., Pommier, Y. *Sequential administration of camptothecin and etoposide circumvents the antagonistic cytotoxicity of simultaneous drug administration in slowly growing human colon carcinoma HT-29 cells.* Eur J Cancer 1992, 28A: 743-8.

42. Pantazis, P., Vardeman, D., Mendoza, J., Early, J., Kozielski, A., DeJesus, A., Giovanella, B. *Sensitivity of camptothecin-resistant human leukemia cells and tumors to anticancer drugs with diverse mechanisms of action.* Leuk Res 1995, 19: 43-55.

43. Pantazis, P., Mendoza, J., DeJesus, A., Early, J., Shaw, M., Giovanella, B. *Development of resistance to 9-nitrocamptothecin by human leukemia U-937 cells in vitro correlates with altered sensitivities to several anticancer drugs.* Anticancer Drugs 1994, 5: 473-9.

44. Pantazis, P., DeJesus, A., Early, J., Rodriguez, R., Chatterjee, D., Han, Z., Wyche, J., Giovanella, B. *Development of human leukemia U-937 cell sublines resistant to doxorubicin: Induction of differentiation and altered sensitivities to topoisomerase-directed drugs.* Anticancer Res 1995, 15: 1873-81.

45. Chaterjee, D., Wyche, J., Pantazis, P. *Induction of apoptosis in malignant and camptothecin-resistant human cells.* Ann NY Acad Sci 1996, 803: 143-56.

46. Beck, W.T. *Unknotting the complexities of multidrug resistance: The involvement of DNA topoisomerases in drug action and resistance.* J Natl Cancer Inst 1989, 81: 1683-5.

47. Tan, K.B., Mattern, M.R., Eng, W.-K., McCabe, F.L., Johnson, R.K. *Nonreproductive rearrangement of DNA topoisomerase I and II genes: Correlation with resistance to topoisomerase inhibitors.* J Natl Cancer Inst 1989, 81: 1732-5.

48. Sugimoto, Y., Tsukahara, S., Oh-hara, T., Liu, L.F., Tsuruo, T. *Elevated expression of DNA topoisomerase II in camptothecin-resistant human tumor cell lines.* Cancer Res 1990, 50: 7962-5.

49. Bartlett, N.L., Wasserman, T.H. *Combined modality therapy.* In: The Chemotherapy Source Book, M.C. Perry (Ed.), Williams & Wilkins, Baltimore, 1996, 109-26.

50. Musk, S.R.R., Steel, G.G. *The inhibition of cellular recovery in human tumor cells by inhibitors of topoisomerase I.* Br J Cancer 1990, 62: 364-7.

51. Boothman, D.A., Wang, M., Schea, R.A., Burrows, N.L., Strickfaden, S., Owens, J.K. *Post-treatment exposure to camptothecin unbalanced the lethal effects of x-rays on radioresistant malignant melanoma cells.* Int J Radiat Oncol Biol Phys 1992, 24: 939-48.

52. Boscia, R.E., Korbut, T., Holden, S.A., Ara, G., Teicher, B.A. *Interaction of topoisomerase I inhibitors with radiation in cis-diaminedichloroplatinum(I)-sensitive and -resistant cells in vitro and in the FSAIIC fibrosarcoma in vivo.* Int J Cancer 1993, 53: 118-23.

53. Falk, S.J., Smith, P.J. *DNA damaging and cell cycle effects of the topoisomerase I poison camptothecin in irradiated human cells.* Int J Radiat Biol 1992, 61: 749-57.

54. Del Bino, G., Bruno, S., Yi, P.N., Darzynkiewicz, Z. *Apoptotic cell death triggered by camptothecin or teniposide. The cell cycle specificity and effects of ionizing radiation.* Cell Prolif 1992, 25: 537-48.

55. Gorczyca, W., Gong, J., Ardelt, B., Traganos, F., Darzynkiewicz, Z. *The cell cycle related differences in susceptibility of HL-60 cells.* Cancer Res 1993, 53: 3186-92.

56. Hennequin, C., Giocanti, N., Balosso, J., Favaudon, Y. *Interaction of ionizing radiation with the topoisomerase I poison camptothecin in growing V-79 and HeLa cells.* Cancer Res 1994, 54: 1720-8.

57. Kirichenko, A.V., Rich, T.A., Newman, R.A., Travis, E.L. *Potentiation of murine MCa-4 carcinoma radioresponse by 9-amino-20(S)-camptothecin.* Cancer Res 1997, 57: 1929-33.

58. Boothman, D.A., Fukunaga, N., Wang, M. *Down-regulation of topoisomerase I in mamalian cells following ionizing radiation.* Cancer Res 1994, 54: 4618-26.

59. Lee, J.M., Bernstein, A. *p53 mutations increase resistance to ionizing radiation.* Proc Natl Acad Sci USA 1993, 90: 5742-6.

60. McIlwrath, A.J., Vasey, P.A., Ross, G.M., Brown, R. *Cell cycle arrest and radiosensitivity of human tumor cell lines: Dependence on wild-type p53 for radiosensitivity.* Cancer Res 1994, 54: 3718-22.

61. Sentman, C.L., Shutter, J.R., Hockenbery, D., Kanagawa, O., Korsmeyer, S.J. *Bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes.* Cell 1991, 67: 879-88.

62. Strasser, A., Harris, A.W., Cory, S. *Bcl-2 transgene inhibits T cell death and perturbs thymic self-censorship.* Cell 1991, 67: 889-99.

63. Han, Z., Chatterjee, D., He, D.-M., Early, J., Pantazis, P., Wyche, J., Hendrickson, E.A. *Evidence for a G_2 checkpoint in p53-independent apoptosis induction by x-irradiation.* Mol Cell Biol 1995, 15: 5849-57.

64. Hahn, G.M. *Thermal enhancement of the actions of anticancer agents.* In: Hyperthermia and Cancer, G.M., Hahn (Ed.), New York, Plenum Publishing Corp., 1992, 55-86.

65. Fisher, G.A., Hahn, G.M. *Enhancement of cis-platinum(II)diaminedichloride cytotoxicity by hyperthermia.* Natl Cancer Inst Monogr 1982, 61: 255-7.

66. Watanabe, N., Niitsu, Y., Umeno, H., Soneda, H., Yamauchi, N., Maeda, M., Urushizaki, I. *Synergistic cytotoxic and antitumor effects of recombinant human tumor necrosis factor and hyperthermia.* Cancer Res 1988, 48: 650-3.

67. Niitsu, Y., Watanabe, N., Umeno, H., Sone, H., Neda, H., Yamauchi, N., Maeda, M., Urushizaki, I. *Synergistic effects of recombinant human tumor necrosis factor and hyperthermia on in vitro cytotoxicity and artificial metastasis.* Cancer Res 1988, 48: 654-7.

68. Tomasovic, S.P., Barta, M., Klostergaard, J. *Neutral red uptake and clonogenic survival assays of the hyperthermia sensitization of tumor cells to tumor necrosis factor.* Radiat Res 1989, 119: 325-37.

69. Fujimoto, S., Konno, O., Kobayashi, K., Kokubun, M., Shrestha, R.D., Kiuchi, S., Takahashi, M., Okui, K. *Augmented antitumor effects of combined treatment with hyperthermia and tumor necrosis factor on human gastric cancer xenografted into nude mice.* Int J Hyperthermia 1991, 7: 511-8.

70. Cohen, J.D., Robins, H.I., Schmitt, C.L. *Tumoricidal interactions of hyperthermia with carboplatin, cisplatin and etoposide.* Cancer Lett 1989, 44: 205-10.

71. Kampinga, H.H., Kruk, G.V.D., Konings, A.W.T. *Reduced DNA break formation and cytotoxicity of the topoisomerase II drug 4'-(9-acridinylamino)methanesulfon-m-anisidide when combined with hyperthermia in human and rodent cell lines.* Cancer Res 1989, 49: 1712-7.

72. Dynlacht, J.R., Wong, R.S.L., Albright, N., Dewey, W.C. *Hyperthermia can reduce cytotoxicity from etoposide without a corresponding reduction in the number of topoisomerase II-DNA cleavable complexes.* Cancer Res 1994, 54: 4129-37.

73. Valenzuela, M.H., Nuñez, M.I., Villalobos, M., Siles, E., McMillan, T.J., Pedraza, V., Ruiz de Almondovar, J.M. *A comparison of p53 and p16 expression in human tumor cells treated with hyperthermia or ionizing radiation.* Int J Cancer 1997, 72: 307-12.

74. Serrano, M., Hannon, G.J., Beach, D. *A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4.* Nature 1993, 366: 704-7.

75. Hinds, P.W., Dowdy, S.F., Eaton, E.N., Arnold, A., Weinberg, R.A. *Function of a human cyclin gene as an oncogene.* Proc Natl Acad Sci USA 1994, 91: 709-13.

76. Teicher, B.A., Holden, S.A., Khandakar, V., Herman, T.S. *Addition of a topoisomerase I inhibitor to trimodality therapy [cis-diaminedichloro-platinum(II)/heat/radiation] in a murine tumor.* J Cancer Res Clin Oncol 1993, 119: 645-51.

77. Pantazis, P., Chatterjee, D., Han, Z., Wyche, J. *Modulation of 9-nitrocamptothecin-induced apoptosis by hyperthermia in human leukemia HL-60 cells.* Anticancer Drugs 1999, 10: 317-22.

78. Pantazis, P., Han, Z., Wyche, J. *Schedule-dependent efficiency of thermochemotherapy in vitro with etoposide and heating at 43 °C.* Anticancer Res 1999, 19: 995-8.

79. *Potential targets for new anticancer drugs.* In: The Anticancer Drugs, W.B. Pratt, R.W. Ruddon, W.D. Ensminger, J. Maybaum (Eds.), Oxford University Press, New York, 1994, 306-36.

80. Warrell, R.P. *Differentiation agents.* In: The Chemotherapy Source Book, M.C. Perry (Ed.), Baltimore, Williams & Wilkins, 1996, 447-58.

81. Scott, R.E. *Differentiation, differentiation/gene therapy and cancer.* Pharmacol Ther 1997, 73: 51-65.

82. Nakaya, K., Chou, S., Kaneko, M., Nakamura, Y. *Topoisomerase inhibitors have potent differentiation-inducing activity for human and mouse myeloid leukemia cells.* Jpn J Cancer Res 1991, 82: 184-91.

83. Chou, S., Kaneko, M., Nakaya, K., Nakamura, Y. *Induction of differentiation of human and mouse myeloid leukemia cells by camptothecin.* Biochem Biophys Res Commun 1990, 1656: 160-7.

84. McSheehy, P.M.J., Gervasoni, M., Lampasona, V., Erba, E., D'Incalci, M. *Studies of the differentiation properties of camptothecin in the human leukaemic cells, K562.* Eur J Cancer 1991, 27: 1406-11.

85. Aller, P., Rius, C., Mata, F., Zorilla, A., Cabañas, C., Bellon, T., Bernabeu, C. *Camptothecin induces differentiation and stimulates the expression of differentiation-related genes in U-937 human promonocytic leukemia cells.* Cancer Res 1992, 52: 1245-51.

86. Singh, S., Razu, U., Mendoza, J., Pantazis, P., Aggarwal, B.B. *Acquisition of cellular resistance to 9-nitrocamptothecin correlates with suppression of transcription factor NF-kappaB activation and potentiation of cytotoxicity by tumor necrosis factor in human histiocytic lymphoma U-937 cells.* Anticancer Drugs 1998, 9: 703-14.

87. Kharbanda, S., Rubin, E., Gunji, H., Hinz, H., Giovanella, B., Pantazis, P., Kufe, D. *Camptothecin and its derivatives induce expression of the c-jun protooncogene in human myeloid leukemia cells.* Cancer Res 1991, 51: 6636-42.

88. Rubin, E., Pantazis, P., Bharti, T., Toppmeyer, D., Giovanella, B.C., Kufe, D. *Identification of a mutant human topoisomerase I*

with intact catalytic activity and resistance to 9-nitrocamptothecin. J Biol Chem 1994, 269: 2433-9.

89. Pantazis, P., Mendoza, J.T., DeJesus, A., Rubin, E., Kufe, D., Giovanella, B. *Partial characterization of human leukemia U-937 cell sublines resistant to 9-nitrocamptothecin.* Eur J Haematol 1994, 53: 135-44.

90. Mitchell, R.I., Tjian, R. *Transcriptional regulation in mammalian cells by senescence-specific DNA binding proteins.* Science 1989, 245: 371-8.

91. Zhang, H., Wang, J.C., Liu, L.F. *Involvement of DNA topoisomerase I in transcription of human ribosomal RNA genes.* Proc Natl Acad Sci USA 1988, 85: 1060-4.

92. Merino, A., Madden, K.R., Lane, W.S., Champoux, J.J., Reinberg, D. *DNA topoisomerase I is involved in both repression and activation of transcription.* Nature 1993, 365: 227-32.

93. Schönfeld, S., Schulz, S., Nyce, J.W. *Effects of inhibitors of topoisomerases I and II on DNA methylation and DNA synthesis in human colonic adenocarcinoma cells in vitro.* Int J Oncol 1992, 1: 807-15.

94. Naveh-Many, T., Cedar, H. *Active gene sequences are undermethylated.* Proc Natl Acad Sci USA 1981, 78: 4246-50.

95. Riggs, A.D., Jones, P.A. *5-Methylcytocine, gene regulation, and cancer*. Adv Cancer Res 1983, 40: 1-30.

96. Gieseler, F., Boege, F., Clark, M., Meyer, P. *Correlation between the DNA-binding affinity of topoisomerase inhibiting drugs and their capacity to induce hematopoietic cell differentiation.* Toxicol Lett 1993, 67: 331-40.

97. Landau, M., Weizman, A., Zoref-Shani, E., Beery, E., Wasserman, L., Landau, O., Gavish, M., Brenner, S., Nordenberg, J. *Antiproliferative and differentiating effects of benzodiazepine receptor ligands on B16 melanoma cells.* Biochem Pharmacol 1998, 56: 1029-34.

98. Yang, Z-Y., Perkins, N.D., Ohno, T., Nabel, E.G., Nabel, G.J. *The p21 cyclin-dependent kinase inhibitor suppresses tumorigenicity in vivo.* Nat Med 1995, 1: 1052-6.

99. Pantazis, P., Chatterjee, D., Han, Z., Wyche, J. *Differentiation of human malignant melanoma cells that escape apoptosis after treatment with 9-nitrocamptothecin in vitro.* Neoplasia 1999, 1: 231-40.

100. Ligget, W.H., Sidransky, D. *Role of the p16 tumor suppressor gene in cancer.* J Clin Oncol 1998, 16: 1197-206.

101. Castellano, M., Gabrielli, B.G., Hussussian, C.J., Dracopoli, N.C., Hayward, N.K. *Restoration of CDKN2A into melanoma cells induces morphologic changes and reduction in growth rate but not anchorage-independent growth reversal.* J Invest Dermatol 1997, 109: 61-8.

102. Pantazis, P., Han, Z., Chatterjee, D., Wyche, J. *Water-insoluble camptothecin analogs as potential antiviral drugs.* J Biomed Sci 1999, 6: 1-7.

103. Pantazis, P. *Camptothecin: A promising antiretroviral drug.* J Biomed Sci 1996, 3: 14-9.

104. Moulton, S., Pantazis, P., Epstein, J.S., Sadaie, M.R. *9-Nitrocamptothecin inhibits in vitro induction of activation of the human immunodeficiency virus type 1 (HIV-1) in latently infected human T lymphocytes.* AIDS Res Hum Retroviruses 1998, 14: 39-49.

105. Sadaie, M.R., Doniger, J., Hung, C-L., Pantazis, P. *9-Nitrocamptothecin (9-NC) selectively inhibits human immunodeficiency virus type 1 replication in freshly infected parental but not 9-NC-resistant U937 monocytoid cells.* AIDS Res Hum Retroviruses 1999, 14: 239-45.

106. Burke, T.G., Mi, Z. *Preferential binding of the carboxylate form of camptothecin by human serum albumin.* Analyt Biochem 1993, 212: 285-7.

107. Mi, Z., Burke, T.G. *Differential interactions of camptothecin lactone and carboxylate forms with human blood components.* Biochemistry 1994, 33: 10325-36.

108. Mi, Z., Burke, T.G. *Marked interspecies variations concerning the interactions of camptothecin with serum albumins: A frequency-domain fluorescence spectroscopic study*. Biochemistry 1994, 33: 12540-5.

109. Wani, M.C., Ronman, P.E., Lindley, L.T., Wall, M.E. *Plant antitumor agents. 18. Synthesis and biological activity of camptothecin analogues.* J Med Chem 1980, 23: 554-60.

110. Giovanella, B.C., Hinz, H.R., Kozielski, A.J., Stehlin, J.S., Silber, R., Potmesil, M. *Complete growth inhibition of human cancer xenografts in nude mice by treatment with 20(S) camptothecin.* Cancer Res 1991, 51: 3052-5.

111. Cao, Z., Harris, N., Kozielski, A., Vardeman, D., Stehlin, J.S., Giovanella, B.C. *Alkyl esters of camptothecin and 9-nitrocamptothecin: Synthesis, in vitro pharmacokinetics, toxicity, and antitumor activity.* J Med Chem 1998, 41: 31-7.

112. Han, Z., Cao, Z., Chatterjee, D., Wyche, J., Pantazis, P. *Propionate and butyrate esters of camptothecin and 9-nitrocamptothecin as antileukemia prodrugs in vitro.* Eur J Haematol 1999, 62: 246-55.

113. Verschraegen, C.F., Natelson, E.A., Giovanella, B.C., Kavanagh, J.J., Kudelka, A.P., Freedeman, R.S., Edwards, C.L., Ende, K., Stehlin, J.S. *A phase I clinical and pharmacological study of oral 9-nitrocamptothecin, a novel water-insoluble topoisomerase I inhibitor.* Anticancer Drugs 1998, 9: 36-44.

114. Verschraegen, C.F., Gupta, E., Loyer, E., Kavanagh, J.J., Kudelka, A.P., Freedman, R.S., Edwards, C.L., Harris, N., Steger, M., Steltz, V., Giovanella, B.C., Stehlin, J.S. *A phase II and pharmacologic study of oral 9-nitrocamptothecin in patients with refractory epithelial ovarian, tubal or peritoneal cancer*. Anticancer Drugs 1999, 10: 375-83.

115. Stehlin, J.S., Giovanella, B.C., Natelson, E.A., De Ipolyi, P.D., Coil, D., Davis, B., Wolk, D., Wallace, P., Trojacek, A. *A study of 9-nitrocamptothecin (RFS-2000) in patients with advanced pancreatic cancer*. Int J Oncol 1999, 14: 821-31.

116. Javaherian, L., Liu, L.F. *Association of eukaryotic DNA topoisomerase I with nucleosomes and chromosomal proteins.* Nucleic Acids Res 1983, 11: 461-72.

117. Rose, K.M., Szopa, J., Han, F.S., Cheng, Y.C., Richter, A., Scheer, U. *Association of DNA topoisomerase I and RNA polymerase I: A possible role for topoisomerase I in ribosomal gene transcription.* Chromosoma 1998, 96: 411-6.

118. Bharti, A.K., Olson, M.O.J., Kufe, D.W., Rubin, E. *Identification of a nucleolin binding site in human topoisomerase I.* J Biol Chem 1996, 271: 1993-7.

119. Simmons, D.T., Melendy, T., Usher, D., Stillman, B. *Simian virus 40 large antigen binds to topoisomerase I.* Virology 1996, 222: 365-74.

120. Haluska, P., Saleem, A., Edwards, T.K., Rubin, E.H. *Interaction between the N-terminus of human topoisomerase I and SV40 large T antigen.* Nucleic Acids Res 1998, 26: 1841-7.

121. Gobert, C., Bracco, L., Rossi, F., Olivier, M., Tazi, J., Lavelle, F., Larsen, A.K., Riou, J.F. *Modulation of DNA topoisomerase I activity by p53.* Biochemistry 1996, 35: 5778-86.

122. Arbor, A., Kaku, S., Kulesz-Martin, M. *Wild-type and mutant forms of p53 activate human topoisomerase I: A possible mechanism for gain of function in mutants.* Cancer Res 1998, 58: 2091-4.

123. Pommier, Y., Kolhagen, G., Wu, C., Simmons, D.T. *Mammalian DNA topoisomerase I activity and poisoning by camptothecin are inhibited by simian virus 40 large T antig*en. Biochemistry 1998, 37: 3818-23.

124. Herben, V.M., Ten-Bokkel Huinink, W.W., Schellens, J.H., Beijnen, J.H. *Clinical pharmacokinetics of camptothecin topoisomerase I inhibitors.* Pharm World Sci 1998, 20: 161-72.

125. Natelson, E.A., Giovanella, B.C., Verschraegen, C.F., Fehir, K.M., Delpolyi, P.D., Harris, N., Stehlin, J.S. *Phase I clinical and pharmacological studies of 20-(S)-camptothecin and 20-(S)-9-nitrocamptothecin as anticancer agents.* Ann NY Acad Sci 1996, 803: 224-30.

126. Lamond, J.P., Wang, M., Kinsella, T.J., Boothman, D.A. *Radiation lethality enhancement with 9-aminocamptothecin: Comparison to other topoisomerase I inhibitors.* Int J Radiat Oncol Biol Phys 1996, 36: 369-76.

127. Kirichenko, A.V., Rich, T.A. *Radiation enhancement by 9-aminocamptothecin: The effect of fractionation and timing of administration.* Int J Radiat Oncol Biol Phys 1999, 44: 659-64.