The Influence of Hyaluronan-CD44 Interaction on Topoisomerase II Activity and Etoposide Cytotoxicity in Head and Neck Cancer

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Objective: To investigate the downstream molecular targets of hyaluronan (HA)-CD44 and phospholipase C (PLC)-mediated calcium ion (Ca^{2+}) signaling in head and neck squamous cell carcinoma (HNSCC). Hyaluronan is a ligand for the CD44 receptor, which interacts with multiple signaling pathways to influence cellular behavior. We recently determined that HA-CD44 interaction promotes PLC-mediated Ca^{2+} signaling and cisplatin resistance in HNSCC.

Design: Proliferation of HNSCC tumor cells and topoisomerase (Topo) II enzymatic activity, including DNA-cleavable complex formation and DNA decatenation, were analyzed in the presence or absence of HA, the Topo II poison etoposide (VP-16), and various inhibitors of PLC and Ca^{2+}-calmodulin kinase II (CaMKII) signaling.

Results: Treatment with HA promoted Topo II phosphorylation, suggesting that HA can modulate Topo II activity. Topoisomerase II–mediated DNA cleavable complex formation was increased by VP-16, and this increase was significantly enhanced by noncytotoxic doses of the PLC inhibitor U73122 and the CaMKII inhibitor KN-62, implicating PLC and CaMKII in Topo II regulation. However, the drug- and inhibitor-mediated increase in DNA cleavable complex formation was reduced with HA pre-treatment. Inhibitors of PLC and CaMKII also enhanced VP-16 inhibition of Topo II–mediated DNA decatenation. Treatment with HA reduced VP-16 cytotoxic activity. On the other hand, U73122 and KN-62 enhanced VP-16 cytotoxic activity and reduced the ability of HA to promote VP-16 resistance.

Conclusion: Our results suggest that HA, PLC, and CaMKII are upstream regulators of Topo II–mediated DNA metabolism in HNSCC and that this signaling pathway could be a promising target for the development of novel therapies against HNSCC.

Arch Otolaryngol Head Neck Surg. 2007;133:281-288

H E A D A N D N E C K S Q U A M O U S c e l l c a r c i n o m a (HNSCC) is the sixth most common cancer worldwide.\(^1\) Advanced-stage HNSCC continues to have poor 5-year survival rates (0%-40%), which have not significantly improved in the last 30 years. Understanding the mechanisms underlying HNSCC tumor proliferation and resistance to standard treatment are critical to improving outcomes for this disease. The interaction between tumor cells and their microenvironment can lead to the activation of oncogenic signaling pathways that promote tumor cell survival.

Hyaluronan (HA) is a glycosaminoglycan component of the extracellular matrix and has well-known biophysical properties. More recently, HA has been studied with regard to its interaction with various cell-signaling pathways.\(^2,3\) Hyaluronan is the primary ligand for the transmembrane receptor CD44, which is expressed in many different normal and malignant cell types.\(^3,6\) In cancer cells, HA interaction with CD44 promotes multiple signaling pathways that influence tumor cell progression behaviors including abnormal growth, migration, and invasion.\(^3,5,8\) The interaction of HA-CD44 with these signaling pathways is incompletely understood, and little is known about the role of HA and CD44 in HNSCC.

In several cell model systems, it has been found that HA-CD44 signaling promotes intracellular calcium ion (Ca^{2+}) mobilization.\(^9,11\) Hyaluronan stimulation of CD44 leads to kinase phosphorylation of phospholipase C (PLC) to its active phosphorylated form. Phosphorylated PLC catalyzes the conversion of phosphatidylinositol-4,5-bisphosphate to inositol-1,4,5-triphosphate (IP_3) and diacylglycerol. Acting as a second messenger, IP_3 binds to the IP_3 receptor to induce the release of Ca^{2+} from intracellular stores. The release of Ca^{2+} from intracellular storage sites is known to me-
mediate a variety of important cell processes, including alterations in cytoskeleton, migration, invasion, and promotion of cell survival.\textsuperscript{9,12} Regulation of calcium signaling has been shown to be important in many cancers.\textsuperscript{12-14} We recently reported that HA-CD44 interaction promotes PLC-mediated Ca\textsuperscript{2+} mobilization and cisplatin resistance in HNSCC.\textsuperscript{14}

In the present investigation, we were interested in examining potential downstream targets of HA-CD44 and PLC-mediated Ca\textsuperscript{2+} mobilization. One of the mechanisms by which intracellular Ca\textsuperscript{2+} may trigger early signal transducing events during HA-CD44-mediated cellular function involves its interaction with calmodulin, a ubiquitous Ca\textsuperscript{2+}-binding protein.\textsuperscript{15,16} It is known that Ca\textsuperscript{2+}-dependent calmodulin is involved in the activation of several important enzymes including Ca\textsuperscript{2+}-calmodulin–dependent kinase II (CaMKII), a serine-threonine protein kinase that is activated by Ca\textsuperscript{2+} and calmodulin to phosphorylate diverse substrates that promote transcriptional activation and cell cycle progression.\textsuperscript{17-20} The effect on downstream targets modulated by CaMKII activation includes inhibition of apoptosis in small-cell lung cancer and increased proliferation in thyroid and glioma cells.\textsuperscript{15,17,18}

DNA topoisomerase (Topo) I and Topo II are enzymes that control and alter the topologic states of DNA in both prokaryotes and eukaryotes.\textsuperscript{19,20} Specifically, Topo I has been shown to alter DNA topology by inducing transient single-stranded breaks in the DNA backbone, whereas Topo II is capable of introducing transient double-stranded DNA breaks and transferring an intact DNA duplex through the break before resealing it.\textsuperscript{19,20} Topo II functions to regulate DNA underwinding and overwinding by catalyzing catenation, decatenation, and relaxation of supercoiled DNA. Topoisomerase II phosphorylation and subsequent function involves its interaction with calmodulin, a ubiquitous Ca\textsuperscript{2+}-binding protein.\textsuperscript{15,16} It is known that Ca\textsuperscript{2+}-dependent calmodulin is involved in the activation of several important enzymes including Ca\textsuperscript{2+}-calmodulin–dependent kinase II (CaMKII), a serine-threonine protein kinase that is activated by Ca\textsuperscript{2+} and calmodulin to phosphorylate diverse substrates that promote transcriptional activation and cell cycle progression.\textsuperscript{17-20} The effect on downstream targets modulated by CaMKII activation includes inhibition of apoptosis in small-cell lung cancer and increased proliferation in thyroid and glioma cells.\textsuperscript{15,17,18}

Although our understanding of Topo II regulation is incomplete, it is thought that Topo II phosphorylation and dephosphorylation likely play an important role in modulating its physiologic activity throughout the cell cycle.\textsuperscript{21-23} Dephosphorylation of Topo II enzymes appears to result in loss of activity, while phosphorylation leads to the stimulation of activity. Evidence linking regulation of Topo II to CaMKII includes in vitro studies demonstrating that activated CaMKII can phosphorylate Topo II and other reports showing that specific CaMKII inhibition diminishes Topo II enzymatic activity.\textsuperscript{23-26} In addition, other kinases, including casein kinase II and protein kinase C, have also been shown to promote stimulation of the catalytic activity of Topo II following its phosphorylation.\textsuperscript{21-23}

The important role of Topo II in DNA metabolism makes it an attractive drug target, and Topo II inhibition is the mechanism for a number of important anticancer agents.\textsuperscript{27} Topoisomerase II poisons kill cancer cells by stabilizing an enzyme-cleaved DNA complex (known as the cleavable complex) that is a transient intermediate in the catalytic cycle of Topo II when the enzyme is covalently attached to the DNA at DNA strand breaks. The cleavable complexes interact with the cell's DNA replication or transcription machinery to cause permanent DNA strand breaks, which trigger the recombination and repair of pathways and mutagenesis. If these breaks overwhelm the cell, they can initiate apoptosis pathways leading to cell death. Etoposide (VP-16) is a Topo II poison capable of stabilizing Topo II–DNA cleavable complexes and inhibiting DNA decatenation, resulting in eventual cell death. Given the central role of Topo II in tumor cell DNA metabolism, it is important to understand the mechanism by which VP-16 and other drugs alter Topo II function in HNSCC.

In the present study, our hypothesis was that HA, PLC, and CaMKII would influence Topo II activity in HNSCC. Our results indicate that HA promotes CD44-dependent Topo II phosphorylation. We also found that specific inhibitors of PLC and CaMKII signaling inhibit HA-mediated Topo II phosphorylation and enhance VP-16–mediated inhibition of Topo II activity, implicating PLC and CaMKII as upstream regulators of Topo II. On the other hand, HA treatment reduced the effect of PLC and CaMKII inhibitors on Topo II–mediated DNA cleavable complex formation activity. Finally, HA treatment decreased VP-16 cytotoxic activity in an HNSCC cell line, while inhibitors of PLC and CaMKII enhanced VP-16 cytotoxic activity.

**METHODS**

CELL CULTURE

The cell line HSC-3 (Japan Cancer Research Resources Bank, Tokyo, Japan) was established in 1985 from a primary oral tongue squamous cell carcinoma removed from a 64-year-old male patient. The HSC-3 cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum. Cells were routinely serum starved (and therefore deprived of the high levels of HA that are present in serum) before adding HA.

**ANTIBODIES AND REAGENTS**

Monoclonal rat antihuman CD44 antibody (clone: 020; isotype: IgG2b), which recognizes a common determinant of the CD44 class of glycoproteins, was obtained from CMB-TECH Inc (San Francisco, Calif). Monoclonal mouse antihuman Topo II antibody, which recognizes the 170-kDa Topo IIa protein, was obtained from BD Transduction Laboratories (San Jose, Calif). Polyclonal rabbit antiphosphoserine antibody, which detects all phosphoserine proteins, was obtained from Chemicon International (Temecula, Calif). The PLC inhibitor U73122 and the CaMKII inhibitor KN-62 ([1-N-(3-isoquinolinylsulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine) were obtained from CalBiochem (La Jolla, Calif). The inhibitors were diluted in dimethyl sulfoxide to the appropriate concentration required for the experiments. For each experiment, dimethyl sulfoxide alone was used to obtain a control baseline for the drug effect. High molecular mass HA polymers (approximately 500 000-Da polymers) obtained from Healon (Pharmacia, Erlangen, Germany) were prepared by gel filtration chromatography using a Sephacryl S1000 column (GE Healthcare Biosciences, Piscataway, NJ). The purity of the high molecular mass HA polymers used in our experiments was further verified by anion-exchange high-performance liquid chromatography. To achieve the specified concentration for the
IMMUNOBLOTTING AND IMMUNOPRECIPITATION TECHNIQUES

After growing in serum-free media for 24 hours, HSC-3 cells were incubated with or without HA (50 µg/mL) for 5 minutes or they were pretreated with anti-CD44 antibody (1:1000), U73122 (5µM), or KN-62 (10µM) for 60 minutes, followed by the addition of HA (3 minutes). Subsequently, cells were solubilized in 50mM HEPES (N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonate]) (pH 7.5), 150mM sodium chloride, 20mM magnesium chloride, 1.0% NP-40, 0.2mM sodium orthovanadate, 0.2mM phenylmethylsulfonyl fluoride, 10 µg/mL of leupeptin, and 3 µg/mL of aprotinin. After brief centrifugation, the samples were first subjected to immunoprecipitation using anti–Topo II antibody followed by incubation with agarose bead-conjugated anti-IgG, electrophoresed on 4% to 12% Tris-glycine gels (Novex, San Diego, Calif), and blotted onto nitrocellulose. After blocking nonspecific sites with 5% milk, the nitrocellulose filters were incubated with antiphosphoserine antibody or with anti–Topo II antibody followed by incubation with horseradish peroxidase-labeled antirabbit IgG. The blots were then developed by the enhanced chemiluminescence system (GE Healthcare Biosciences).

MEASUREMENT OF TOPO II–MEDIATED DNA CLEAVABLE COMPLEX FORMATION

The effect of different inhibitors on VP-16–stabilized Topo II–mediated DNA cleavable complex formation was determined by the sodium dodecyl sulfate/potassium chloride precipitation technique, as previously described. Briefly, HSC-3 cells (4 x 10^6) were labeled with 0.2 µCi/mL (7.4 kBq/mL) of [14C]-leucine and 0.6 µCi/mL (22.2 kBq/mL) of (methyl-3H) thymidine for 24 hours and treated with or without HA (50 µg/mL) and the PLC inhibitor U73122 (5µM) or the CaMKII inhibitor KN-62 (10µM). Next, the cells were treated with VP-16 (1µM) for 1 hour at 37ºC, washed in cold phosphate-buffered saline, and lysed at 65ºC in a solution of 1.25% 5mM EDTA and 0.4 mg/mL of denatured herring sperm. The lysates were then incubated at 65ºC for 10 minutes, brought to 65mM potassium chloride, and placed on ice for 10 minutes. A precipitate formed, which was collected by centrifugation, washed and resuspended in water, and transferred to a liquid scintillation vial which was collected by centrifugation, washed and resuspended in water, and transferred to a liquid scintillation vial for determination of the radioactivity in the pellet. The ratio of the amount of [14C]-DNA to [3H]-protein reflects the Topo II–DNA cleavable complex formation and was determined for each experimental condition. The amount of Topo II–DNA cleavable complexes for the control condition (no VP-16, inhibitor, or HA) was arbitrarily assigned the value of 1.0, and the amount of Topo II–DNA cleavable complexes for each of the experimental conditions was expressed relative to the control condition. Each experiment was repeated at least 3 times.

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RESULTS

HA PROMOTES CD44-DEPENDENT TOPO II PHOSPHORYLATION

Topo II activity is thought to be regulated through phosphorylation of serine residues. To address the question of whether Topo II phosphorylation is sensitive to HA–CD44 signaling in HNSCC, we first carried out immunoprecipitation of HSC-3 cells with anti–Topo II antibody, followed by immunoblotting with antiphospho-
serine antibody (Figure 1A) or anti–Topo II antibody (Figure 1B). Our results demonstrate that HA treatment (Figure 1A, lane 2) results in an obvious increase in the amount of serine phosphorylation present in the anti–Topo II antibody–immunoprecipitated materials (reblotted with anti–Topo II antibody for loading control) (Figure 1B, lane 2) compared with the low level of serine phosphorylation (Figure 1A, lane 1) of the Topo II protein present in untreated cells (Figure 1B, lane 1). When cells were pretreated with anti–CD44 antibody (Figure 1A, lane 3), elimination of HA-mediated increased serine phosphorylation in Topo II protein was seen (Figure 1B, lane 3). These results provide evidence that Topo II phosphorylation is sensitive to HA–CD44 signaling. In addition, when cells were pretreated with U73122 (Figure 1A, lane 4) or KN-62 (Figure 1B, lane 5), elimination of HA-mediated increased serine phosphorylation in Topo II protein (Figure 1B, lanes 4 and 5) suggests that PLC and CaMKII are regulators of HA-mediated Topo II activation.

PLC AND CAMKII INHIBITION INCREASES TOPO II–MEDIATED DNA CLEAVABLE COMPLEX FORMATION ACTIVITY

To determine whether HA, PLC, and/or CaMKII regulate Topo II activity, we examined the effect of PLC and CaMKII inhibitors on VP-16–mediated inhibition of Topo II activity. VP-16 is a Topo II poison thought to act by stabilizing the Topo II–DNA cleavable complex, resulting in initiation of apoptosis and cell death. Inhibition of Topo II activity is suggested by an increase in the amount of stabilized Topo II–DNA cleavable complex. HSC-3 cells were labeled with [3H]-DNA and [14C]-protein to determine the amount of Topo II–mediated DNA cleavable complex formation in the presence or absence of HA (50 µg/mL), VP-16 (1µM), U73122 (5µM), and KN-62 (10µM) (Figure 2). The rise in Topo II–mediated DNA cleavable complex formation seen with VP-16 treatment is significantly increased (P = .01) when VP-16 is combined with noncytotoxic doses of the PLC and CaMKII inhibitors U73122 and KN-62, respectively. However, this increased inhibition of Topo II activity is significantly reduced (P = .03) when the inhibitor and Topo II poison treatment is combined with HA. These results suggest that PLC and CaMKII regulate Topo II activity, and this regulatory activity is sensitive to HA signaling.

PLC AND CAMKII INHIBITION DECREASES TOPO II–MEDIATED DNA DECATION

We next examined Topo II–mediated DNA decatenation. Double-stranded DNA cleavage was detected using an agarose gel system to demonstrate the linearization of supercoiled kDNA by Topo II–containing nuclear extracts in the presence or absence of HA (50 µg/mL), VP-16 (1µM), U73122 (5µM), and KN-62 (10µM). Figure 3.
shows an agarose gel used to assess Topo II-mediated kDNA decatenation. The visualized catenated and decatenated products were quantified to calculate the fraction of strand passage, which reflects the ability of Topo II to decatenate kDNA, allowing passage through the gel. The Topo II–mediated decatenation activity when the extracts were treated with VP-16 alone and VP-16 plus nontoxic doses of U73122 or KN-62 was determined, as described in the “Methods” section (Table 1). In comparison with control (no treatment), we found that VP-16 treatment resulted in a 21% increased inhibition of Topo II–mediated kDNA decatenation by VP-16 was enhanced 1.7-fold and 1.9-fold when VP-16 was combined with U73122 or KN-62, respectively; these differences were statistically significant (P = .03). The DNA decatenation assay results provide further evidence for the role of PLC and CaMKII as upstream regulators of Topo II activity. Treatment with HA led to significantly greater Topo II–mediated decatenation activity in the control and VP-16 alone conditions (P = .04). There was slightly less Topo II–mediated decatenation activity with HA treatment in the VP-16 plus inhibitors conditions; however, statistical analysis revealed that these differences were not significant (P = .08), and we conclude that HA does not significantly modulate U73122 and KN-62 inhibition of Topo II DNA decatenation activity.

**HA PROMOTES CD44-DEPENDENT DECREASE IN VP-16 CYTOTOXIC ACTIVITY**

We have previously reported that HA promotes a CD44-dependent decrease in cisplatin, methotrexate, and doxorubicin hydrochloride cytotoxic activity in HNSCC. To determine the role of HA signaling on VP-16–mediated cytotoxic activity, HSC-3 tumor cell growth with VP-16 was measured via MTT assay in the presence or absence of HA and topoisomerase II poison etoposide (VP-16) plus various inhibitors. Topoisomerase II decatenation activity for nuclear extracts without any treatment has been designated as 100%.

![Figure 4](https://example.com/fig4.png)

**Figure 4.** Analysis of topoisomerase II poison etoposide (VP-16) cytotoxic activity. HSC-3 cells grown in serum-free media were treated with VP-16 in the presence or absence of hyaluronan (HA) (50 µg/mL) or anti-CD44 antibody plus HA. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were performed. Error bars represent calculated standard error of the means. The graph displays cell growth as percentage of control (cell growth of HSC-3 cells without HA and VP-16 treatment). IC<sub>50</sub> indicates 50% inhibitory concentration.

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<thead>
<tr>
<th>Treatment</th>
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<tr>
<td>Control (no treatment)</td>
<td>100</td>
<td>108</td>
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<tr>
<td>VP-16</td>
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<tr>
<td>VP-16 + KN-62</td>
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**Table 1. Measurement of Topoisomerase (Topo) II–Mediated Decatenation Activity**

Abbreviation: HA, hyaluronan.

Values represent the mean of at least 3 experiments. Topoisomerase II–mediated decatenation activity, determined as described in the “Methods” section, is reported for nuclear extracts treated with or without HA and topoisomerase II poison etoposide (VP-16) plus various inhibitors. Topoisomerase II decatenation activity for nuclear extracts without any treatment has been designated as 100%.

**Table 2. Measurement of HA-CD44–Dependent VP-16 Cytotoxic Activity in HSC-3 Cells**

Abbreviations: HA, hyaluronan; IC<sub>50</sub>, 50% inhibitory concentration.

To investigate whether VP-16 cytotoxic activity in HNSCC might also be regulated by PLC and CaMKII signaling, we performed MTT assays with HSC-3 cells in the presence of increasing concentrations of VP-16 combined with inhibitors of PLC and CaMKII. At a concentration of 5µM, the PLC inhibitor U73122 alone caused minimal inhibition of tumor cell proliferation (Figure 5A). However, the same concentration of U73122 when combined with VP-16 significantly enhanced VP-16 cytotoxic activity (P<.01) (Figure 5A and Table 2). Since CaMKII is activated by intracellular Ca<sup>2+</sup> signaling, we next used the CaMKII inhibitor KN-62. At a concentration of 10µM, KN-62 alone caused minimal inhibition of tumor cell proliferation.

**PLC AND CAMKII INHIBITION ENHANCES VP-16 CYTOTOXIC ACTIVITY**
Figure 5. Analysis of phospholipase C and calcium ion (Ca^{2+})-calmodulin kinase II inhibitors and topoisomerase II poison etoposide (VP-16) cytotoxic activity. A, HSC-3 cells grown in serum-free media were treated with VP-16 in the presence or absence of hyaluronan (HA) (50 µg/mL) plus U73122 (5µM). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were performed. Error bars represent calculated standard error of the means. KN-62 alone had little effect on growth inhibition at a concentration of 10µM (inset). B, HSC-3 cells grown in serum-free media were treated with VP-16 in the presence or absence of HA (50 µg/mL) plus U73122 (5µM). MTT assays were performed. Error bars represent calculated standard error of the means. U73122 alone had little effect on growth proliferation at a concentration of 5µM (inset). B, HSC-3 cells grown in serum-free media were treated with VP-16 in the presence or absence of hyaluronan (HA) (50 µg/mL) plus U73122 (5µM). MTT assays were performed. Error bars represent calculated standard error of the means. U73122 alone had little effect on growth inhibition at a concentration of 10µM (inset).

We have previously demonstrated that HA-CD44 signaling promotes PLC-mediated Ca^{2+} mobilization in HNSCC. We sought to determine whether Topo II phosphorylation and activity were sensitive to HA signaling in HNSCC. The PLC inhibitor U73122 and the CaMKII inhibitor KN-62 were used to determine the role of PLC and CaMKII in the regulation of Topo II in HSC-3 cells. Our data indicate that specific inhibitors of PLC and CaMKII signaling increase VP-16–mediated inhibition of Topo II activity and enhance VP-16 cytotoxic activity. We also found that HA promotes Topo II phosphorylation, decreases the impact of PLC and CaMKII inhibitors on Topo II–mediated DNA cleavable complex formation activity, and reduces VP-16 cytotoxic activity in HSC-3 cells. These results suggest that HA, PLC, and CaMKII are upstream regulators of Topo II. The role of Topo II in cancer growth and resistance to treatment has been studied extensively; however, we believe that this is the first study linking HA-CD44 signaling to Topo II activity.

Figure 6 represents our proposed model for the interaction of hyaluronan (HA)-CD44 signaling, phospholipase C (PLC)-mediated calcium ion (Ca^{2+}) mobilization, and topoisomerase (Topo) II activity in head and neck squamous cell carcinoma (HNSCC). Specifically, we propose that HA interaction with CD44 promotes PLC-mediated Ca^{2+} mobilization, Ca^{2+}-calmodulin kinase II (CaMKII) activation, increased Topo II phosphorylation, and enhanced Topo II–mediated DNA metabolism. Our data suggest that increased Topo II–mediated DNA metabolism results in improved HNSCC tumor cell survival in the presence of the Topo II poison etoposide (VP-16). P indicates phosphorylated.
Our use of U73122 and KN-62 to selectively inhibit PLC and CaMKII, respectively, is well-supported in the literature.\textsuperscript{14-17,24-26,31,32} U73122 inhibits all isoforms of PLC but not other phospholipases.\textsuperscript{33} KN-62 does not affect protein kinase C, protein kinase A, CaMKIV, or cyclic adenosine monophosphate–dependent protein kinase II.\textsuperscript{32} Therefore, at the concentration range used in our study, we expect that these 2 inhibitors would produce selective inhibition of the intended enzymatic activity. Thus, the elimination of HA-mediated Topo II phosphorylation and VP-16 resistance with U73122 and KN-62 treatment supports the hypothesis that PLC and CaMKII regulate HA-mediated Topo II activity in HNSCC.

The DNA cleavable complex and decatenation assays were chosen because we wished to selectively study Topo II activity. Unknotting (such as decatenation) and DNA cleavage (such as DNA cleavable complex formation) assays are specific for Topo II function, whereas relaxation and nicking assays are specific for Topo I activity.\textsuperscript{19,20} Our data regarding the HA-protective effect on U73122 and KN-62 inhibition of Topo II activity do contain inconsistency, which we cannot fully explain. Hyaluronan diminished the effect of PLC and CaMKII inhibitors on DNA cleavable complex formation but did not diminish their effect on DNA decatenation activity and on VP-16 cytotoxic activity. One possibility is that the various Topo II–mediated activities, such as decatenation and DNA cleavable complex formation, may be differentially affected by HA signaling. Perhaps the influence of HA treatment on DNA cleavable complex formation is relatively greater than the influence of the 2 inhibitors, whereas for DNA decatenation activity or VP-16 cytotoxic activity, the inhibitor effect is greater than the HA effect.

Tumor resistance is a limiting factor in the usefulness of all Topo II–targeting drugs. Resistance to Topo II poisons has been associated with the broad drug-resistant phenotype defined as multidrug resistance and is hypothesized to be mediated through overexpression of a membrane glycoprotein, PGP.\textsuperscript{24-27} Reduced drug accumulation in multidrug-resistant cells is suggested to involve drug efflux through increased membrane pump activity. However, resistance to Topo II–targeting drugs unrelated to drug accumulation defects has been identified.\textsuperscript{24-26} Other mechanisms of resistance to Topo II inhibition include changes in Topo II protein levels and alterations in the Topo II protein itself. Alterations to Topo II could involve gene mutations as well as posttranslational modifications such as changes in phosphorylation state. The role of phosphorylation state of Topo II in governing drug-stimulated inhibition of DNA metabolism remains controversial, with studies showing both hyperphosphorylation and hypo-phosphorylation associated with enhanced VP-16 resistance.\textsuperscript{22,24,26} Our data are consistent with a previous study that demonstrated that increased phosphorylation of Topo II by casein kinase resulted in increased Topo II activity, decreased DNA cleavable complex formation, and increased VP-16 resistance.\textsuperscript{22}

Although VP-16 was previously not found to be effective as a single agent against head and neck cancer, it has recently been shown to have efficacy when given in combination with radiation therapy for advanced, unresectable head and neck cancer.\textsuperscript{33,34} One can speculate, based on our data, that HA-CD44 signaling and its promotion of Topo II activity may explain the typical resistance of head and neck cancers to Topo II inhibitors such as VP-16. Furthermore, our data suggest that targeted inhibition of HA-CD44–mediated Topo II activity in combination with VP-16 or other Topo II inhibitors might have clinical utility, possibly as a radiation sensitizer for treatment-resistant head and neck cancers.

One of the common mechanisms for tumor drug resistance is through increased DNA damage repair. Topoisomerase II is an essential nuclear enzyme that is a key component in DNA transcription and replication, maintenance of the nuclear scaffold, and other critical aspects of chromosome dynamics. We have previously found that HA-CD44 interaction is capable of promoting resistance to multiple chemotherapeutic agents in HNSCC.\textsuperscript{8,14} Although Topo II–targeting drugs have yet to find extensive use in HNSCC, it is interesting to speculate whether HA promotion of Topo II activity could facilitate tumor cell survival in the presence of drug agents not typically associated with direct Topo II inhibition. Our findings suggest that therapeutic targeting of Topo II–associated signaling pathways in HNSCC as a strategy for sensitizing tumors to chemotherapeutic agents may be worth exploration. We are currently investigating the potential role of Topo II activity in HA-CD44–mediated tumor resistance to other chemotherapy agents.

Because the p53 tumor suppressor gene is known to play an important role in HNSCC development and treatment resistance, one may consider the potential effects of p53 mutation on our model. In one study, p53-negative ovarian cells were found to be more resistant to VP-16 and underwent less apoptosis than p53-positive cells.\textsuperscript{35} In a second study, a Topo II inhibitor caused glioma cells to undergo apoptosis and have increased expression of p53.\textsuperscript{36} These 2 studies suggest that p53 modulates anti-Topo II drug activity. We have not directly examined the p53 status of our cell system; however, the HSC-3 cell line has been reported to express a mutant p53 with C-terminal truncation of the protein.\textsuperscript{37} If our HSC-3 cells have p53 mutations, the ability to induce increased VP-16 resistance through HA treatment is intriguing.

In summary, HA promoted Topo II phosphorylation, while inhibitors of PLC and CaMKII were shown to eliminate HA-mediated Topo II phosphorylation and reduce Topo II activity. Hyaluronan also promoted VP-16 resistance, while perturbation of PLC and CaMKII signaling led to enhanced etoposide cytotoxic activity. Our results suggest that HA, PLC, and CaMKII are upstream regulators of Topo II–mediated DNA metabolism in HNSCC and suggest that this signaling pathway could be a promising target for development of novel therapies against HNSCC.
ritiy of the data and the accuracy of the data analysis. Study concept and design: Wang and Bourguignon. Acquisition of data: Wang and Peyrollier. Analysis and interpretation of data: Wang, Peyrollier, and Bourguignon. Drafting of the manuscript: Wang and Peyrollier. Critical revision of the manuscript for important intellectual content: Wang and Bourguignon. Obtained funding: Wang and Bourguignon. Administrative, technical, and material support: Bourguignon. Study supervision: Bourguignon.

Financial Disclosure: None reported.

Funding/Support: This work was supported by a Northern California Institute for Research and Education Young Investigator Award, American Academy of Otolaryngology—Head and Neck Surgery/American Head and Neck Society Young Investigator Award, US Public Health Services grants PO1 AR39448, RO1 CA66163, and RO1 CA78633 from the National Institutes of Health, and a Department of Veterans Affairs (VA) Merit Review grant. Dr Bourguignon is a VA career scientist.

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