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Ellagic Acid Inhibits Nucleoside Diphosphate Kinase-B Activity

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Despite decades of research and significant advances in the detection and treatment of primary breast carcinoma, patients who succumb to the disease do so because of the formation of metastatic tumors. Metastasis is a complex cascade of events involving proteolysis, tumor cell motility, intravasation and extravasation, proteolysis, tumor growth, and angiogenesis. The so-called metastasis suppressor gene, Nm23, may be an integral mediator involved in one or more of these events. Expression of two human isoforms, Nm23-H1 and Nm23-H2, is reported to be inversely associated with the metastatic potential of a variety of cancers [1-3]. The gene products, NDPK-A and NDPK-B, of the two Nm23 genes were named for their function as isoforms of the enzyme nucleoside diphosphate (NDP) kinase. These enzymes, in the presence of divalent cations, covalently transfer the terminal γ-phosphate of a nucleoside triphosphate to a nucleoside diphosphate via a high-energy phosphohistidine intermediate.

$$N_1DP + N_2TP \Leftrightarrow N_1TP + N_2DP$$

We have previously reported that both NDPK-A (H1) and NDPK-B (H2) are expressed as *ecto* enzymes and that NDPK-B is elaborated into the extracellular environment by the breast carcinoma cell line MDA-MB-435s [4]. The presence of NDP kinase activity on the surface and in the external environment of cancer cells provides an effective mechanism for generating extracellular ATP. The localized production of extracellular ATP by tumor derived NDPK-B may facilitate the process of metastasis as it may support tumor cell transit, intravasation and angiogenesis. Indeed, the fact that extracellular ATP plays a significant role in the local regulation of blood flow [5] and that nucleotides inhibit platelet aggregation and stimulate endothelial cell contraction [6] is consistent with these events. Thus, inhibitors of NDPK-B may potentiate the suppression of metastasis.

It was immediately evident that no specific, potent inhibitors for nucleoside diphosphate kinases were known. We thus focused our search in a hypothesis directed manner by examining compounds that had been described as having anti-angiogenesis or general anti-cancer properties. As inhibitor data were gathered, subsequent compounds were chosen based on conserved structural motifs found in the most potent inhibitors. Currently, the structure-based literature search approach has been exhausted. We are now progressing to strategies based on molecular modeling to predict novel compounds that may inhibit NDPK-B kinase activity.

The compounds selected for study were chosen for a variety of reasons. The polyphenolic tea compounds (theaflavins, EGCG, EGC, and ECG) are known to suppress cancer cell proliferation, inhibit invasion into Matrigel®, and inhibit angiogenesis [7-12]. Piceatannol, resveratrol, genestein, and silymarin are also polyphenolic compounds possessing anticarcinogenic properties via a variety of proposed mechanisms, including inhibition of COX and LOX pathways [13-15]. They were also chosen because of conserved structural motifs between them and the tea compounds. Purpurogallin was originally studied as a potential inhibitor of oncogene product enzyme activity [16]. The nucleoside analogs AZT, PAPS, and 8-Cl-cAMP were chosen as potential NDPK-B inhibitors because of previous reports of their inhibition of nucleoside diphosphate kinases in relation to their anti-HIV properties [17-19] and because their relatively high K_is could be compared to more promising compounds. Ellagic acid was originally identified through a structure search based on the apparent importance of the gallate moiety contained in the most potent polyphenolic tea compounds. It was later reported as a potential chemopreventative agent [20]. Interestingly, we find that the most potent inhibitors of NDPK-B activity have the least defined mechanism of tumor growth or angiogenesis inhibition.

METHODS:

Production of NDPK-B. MDA-MB-435s breast ductal carcinoma cells were grown to confluency in DMEM with 10% heat-inactivated FBS and antibiotics. Medium was replaced with Krebs buffer (25 mM Hepes, pH 7.4) and incubated at 37°C with gentle rocking for 4 h. The supernatant was removed and concentrated. NDPK activity of the retentate was assayed and stored at -20°C.

NDPK-B kinase activity assay. Conversion of ADP to ATP was quantified using the Luciferin-Luciferase ATP assay (Sigma). All necessary steps were taken to reduce or eliminate interference between potential NDPK-B inhibitors and the assay.

RESULTS: NDPK-B activity (Table 1) is inhibited by the polyphenolic constituents of tea (EGCG, ECG, and the theaflavins). The nucleoside analogs, 8-Cl-cAMP and PAPS, inhibit NDPK-B transphosphorylation activity but

with relatively low potency (Table 1). The compound ellagic acid (hexahydroxydiphenic acid dilactone), found through a structure search based on the conserved moiety contained in the most potent NDPK-B inhibiting polyphenolic tea compounds, has the highest affinity of NDPK-B thus far tested (Table 1).

Table 1. Inhibitors of NDPK-B activity

Compound	Inhibition	Concentrations Tested	IC_{50}	Inhibition Type	K_m(ADP)
EGCG	Yes	3-700 μM	150 μM	NC	9.67 ± 1.75
ECG	Yes	3-700 μM	170 μM	NC	13.34 ± 1.70
EGC	No	3-700 μM	-	-	-
Black tea extract	Yes	0.7-300 μg/ml	80 μg/ml	NC	11.35 ± 2.75
Piceatannol	No	1-1000 μM	-	-	-
Resveratrol	No	1-1000 μM	-	-	-
Genestein	No	0.3-30 μM	-	-	-
Silymarin	No	3-1000 μg/ml	-	-	-
Purpurogallin	Yes	1-300 μM	600 μM	NC	20.44 ± 3.54
AZT	No	0.3-100 μM	-	-	-
PAPS	Yes	10-1000 μM	500 μM	NC	7.14 ± 1.38
8-Cl-cAMP	Yes	3-1000 μM	1 mM	NC	7.54 ± 1.52
Ellagic Acid	Yes	1-300 uM	23 uM	NC	18.80 ± 2.66

Partially purified NDPK-B, secreted from tumor cells, was incubated with ADP and GTP in the presence of varying concentrations of putative NDPK inhibitors or putative metastasis or angiogenesis inhibitors and the resulting ATP generated, measured by chemiluminescence using the luciferin-luciferase assay. All compounds found to inhibit NDPK-B did so by depressing the V_{max} of the enzyme while maintaining a statistically similar K_M, suggesting non-competitive inhibition with respect to phosphoryl donor (GTP). Molecular modeling studies support these kinetic assumptions (not shown).

DISCUSSION: Breast cancer cells translate Nm23-H2 as an exo-enzyme, NDPK-B. The enzyme is secreted as a phosphoprotein and is capable of transphosphorylation activity in the absence of a phosphoryl donor (Anzinger *et al*., these *Proceedings*). This activity may be a mechanism for producing elevated extracellular ATP, particularly in the setting of angiogenesis required for the growth of breast cancer metastases. Several compounds reported to possess anti-angiogenic or anti-tumorigenic properties inhibit NDPK-B activity. Indeed, a trend was found where compounds with more thoroughly described mechanisms of anti-tumorigenicity tended to inhibit NDPK-B activity less or not at all while compounds with poorly described mechanisms of anti-tumorigenicity tended to inhibit NDPK-B activity potently. The anti-NDP kinase property reported here suggests a novel mechanism by which these compounds may be anti-tumorigenic. Taken together, these findings suggest the hypothesis that inhibition of NDPK-B activity is mechanistically associated with inhibition of metastasis by breast cancer cells. Ellagic acid, along with the other potent NDPK inhibitors, will provide the necessary data for future predictive studies aimed at the design of novel NDPK-B inhibitors.

CONCLUSION: NDPK-B secretion from breast cancer cells and its action to produce ATP extracellularly leads us to believe that the hypothesis of Nm23 gene expression being directly correlated with low metastasis potential is an incomplete assessment. Indeed, the fact that a number of compounds that are claimed to be chemopreventative agents inhibit NDPK activity could further implicate extracellular nucleotides in the metastatic process. There are reports of tumorogenesis studies that contradict the original claims of non-metastatic potential [21] now ascribed to Nm23 [22]. We have examined a list of compounds implicated in whole tumor and angiogenesis inhibition and find some to be effective NDPK-B inhibitors. Our data can now be used in predicative models to design novel inhibitors of NDPK-B activity. Ultimately, novel potent NDPK-B inhibitors can be tested in *in vivo* whole tumor growth and angiogenesis experiments.

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