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Original Contribution

Chemotherapeutic potential of diazeniumdiolate-based aspirin prodrugs in breast cancer

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ABSTRACT

Diazeniumdiolate-based aspirin prodrugs have previously been shown to retain the anti-inflammatory properties of aspirin while protecting against the common side effect of stomach ulceration. Initial analysis of two new prodrugs of aspirin that also release either nitroxyl (HNO) or nitric oxide (NO) demonstrated increased cytotoxicity toward human lung carcinoma cells compared to either aspirin or the parent nitrogen oxide donor. In addition, cytotoxicity was significantly lower in endothelial cells, suggesting cancer-specific sensitivity. To assess the chemotherapeutic potential of these new prodrugs in treatment of breast cancer, we studied their effect both in cultured cells and in a nude mouse model. Both prodrugs reduced growth of breast adenocarcinoma cells more effectively than the parent compounds while not being appreciably cytotoxic in a related nontumorigenic cell line (MCF-10A). The HNO donor also was more cytotoxic than the related NO donor. The basis for the observed specificity was investigated in terms of impact on metabolism, DNA damage and repair, apoptosis, angiogenesis and metastasis. The results suggest a significant pharmacological potential for treatment of breast cancer.

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are used extensively for treatment of pain, inflammation, and fever [1]. Daily intake of aspirin also reduces the risk of cardiovascular disease [2,3], however with concomitant increases in the incidence of a variety of adverse side effects including gastrointestinal bleeding and ulceration [3–6]. Diverse structural modifications have been employed to improve the benefit versus risk profile of NSAIDs. For instance, increased steric bulk has been shown to provide protection against the gastrointestinal irritation that is common in the use of traditional NSAIDs [7–10] by improving specificity toward cyclooxygenase (COX)-2 over COX-1 [11]. However, the cardioprotection observed for aspirin is not replicated by other NSAIDs, as chronic use increases the risk of myocardial infarction and stroke [12].

Another synthetic strategy that has been employed for improving the NSAID safety profile is to couple other moieties to NSAIDs to produce prodrugs with multiple functionalities. For example, recent attention has been given to nitric oxide (NO) derivatives (NO-NSAIDs) [13–15], which are designed to harness the beneficial cardiovascular and gastrointestinal effects of NO (e.g., regulation of blood pressure, inhibition of platelet aggregation, repair of mucosal injury) [16–19].

Abbreviations: A549, human alveolar epithelial carcinoma cells; COX, cyclooxygenase; DCF, 2′,7′-dichlorofluorescein; DCF-2DA, 4-amino-5-methylamino-2′,7′-dichlorofluorescein diacetate; DEA/NO, sodium 1-(*N*,*N*-diethylamino)diazen-1-ium-1,2-diolate NONOate, diazeniumdiolate; DEA/NO-aspirin, O^2-(acetylsalicyloyloxymethyl)-1-(*N*,*N*-diethylamino)-diazen-1-ium-1,2-diolate; DMSO, dimethyl sulfoxide; E-cadherin, epithelial cadherin; ER(−), estrogen receptor α-negative; ER(+), estrogen receptor α-positive; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; HIF, hypoxia-inducible factor; HNO, nitroxyl; HPRT, hypoxanthine phosphoribosyltransferase; HUVECs, human umbilical vein endothelial cells; iNOS, inducible isoform of NO synthase; IPA/NO, sodium 1-(*N*-isopropylamino)diazen-1-ium-1,2-diolate; IPA/NO-aspirin, O^2-(acetylsalicyloyloxymethyl)-1-(*N*-isopropylamino)-diazen-1-ium-1,2-diolate; MCF7, human ER(+) breast adenocarcinoma cells; MCF-10A, nontumorigenic, human epithelial breast cells; MDA-MB-231, human ER(−) breast adenocarcinoma cells; MDA-MB-468, human ER(−) breast adenocarcinoma cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO, nitric oxide; NSAID, nonsteroidal anti-inflammatory drug; NO-NSAID, NO-donating NSAID derivative; NONO-NSAID, diazeniumdiolate-NSAID adduct; NONO-aspirin, diazeniumdiolate-aspirin adduct; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; PGE_2, prostaglandin E_2; RNS, reactive nitrogen species; ROS, reactive oxygen species; RPMI, Roswell Park Memorial Institute; RT-PCR, real-time polymerase chain reaction; SERM, selective estrogen receptor modulator; VEGF, vascular endothelial growth factor

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Such adducts do in fact retain the anti-inflammatory and analgesic properties of the NSAID while reducing gastrointestinal, cardiovascular, and renal side effects in animal models (see [15]). A significantly lower degree of gastric injury has also been apparent in clinical trials [15,20].

NO-NSAIDs are formulated by covalently attaching NSAIDs to nitrate esters [21], diazeniumdiolates (also called as NONOates) [22,23] and other NO donors [24]. Interestingly, the covalent linker itself has been suggested to reduce NSAID toxicity by increasing stability and absorption rate of the prodrug in the gastrointestinal tract [25]. The linker can also augment localized delivery of NO [26,27], which is a critical feature in NO donor-based treatment of conditions other than hypertension. Cleavage of the linker may also produce bioactive agents.

The prodrug strategy with respect to NO donors was initially developed by Keefer and colleagues who derivatized diazeniumdiolates in the early 1990s [28]. Esterase-sensitive derivatives were later found to significantly decrease proliferation of leukemia cells compared to the parent donor [29]. Incorporation by design of a thiol-sensitive functionality to produce a construct named JS-K significantly enhanced antiproliferative activity [30] and opened up a new research area in the expansive arena of diazeniumdiolates as drug candidates.

The role of NO in cancer is complicated in that NO production can be mutagenic yet can affect apoptosis, proliferation, migration, adhesion, angiogenesis, and vascular permeability [31–33]. Often, low levels of NO are suggested to be protumorigenic, while production of higher, sustained levels of NO can have cytostatic and cytotoxic effects on cancer cells [34,35]. However, expression of the inducible isoform of NO synthase (iNOS) has been reported in malignancies of the breast, prostate, lung, brain, and colon [36–45]. Detection of increased iNOS levels also predicts poor survival in estrogen receptor α-negative (ER(−)) breast cancer patients [46]. Thus, inhibition of iNOS within cancer cells may be therapeutic [32] while delivery of exogenous NO may initiate tumor regression in simulation of the immune system. NO donors have in fact been shown to lead to chemo- [47,48] and radiosensitization [49–51] and to overcome drug resistance by tumor cells [52].

COX-2 expression [53] predicts poor outcome similarly to iNOS, indicating that inflammation is a major driving force in both oncogenesis and resistance to conventional therapeutic regimes [54]. Epidemiological studies show that daily intake of NSAIDs, primarily aspirin, reduces risk of colon, lung, prostate, esophageal, stomach, ovarian, and breast cancer [55–60]. Initial studies of malignant melanoma, Hodgkin's disease, and adult leukemia also found NSAIDs to be protective [61–63]. Early analysis also indicates that NO-NSAIDs inhibit cancer growth [64,65].

Recently nitroxyl (HNO), which is one electron more reduced than NO, has emerged as a biologically relevant nitrogen oxide [66], with promise for the treatment of cardiovascular diseases such as heart failure and ischemic reperfusion injury [67–69]. HNO donors have also been used clinically in the treatment of alcoholism [70,71]. Although HNO has yet to be demonstrated to be endogenously produced, a role in inhibition of tumor development by HNO donors is emerging [72]. In particular, the thiophilicity of HNO [73–75] leads to irreversible thiol modification of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [76,77]. This in turn results in inhibition of glycolysis, which occurs at a substantially accelerated rate in hypoxic, solid tumors compared to normal cells [78].

Other critical thiol-containing enzymes that facilitate cancer progression may also be targets of HNO-donating anticancer agents. Consistent with this idea, HNO has been shown to inhibit breast cancer [72] and neuroblastoma [79] proliferation in mouse xenografts as well as in culture, through increased apoptosis [72]. In addition to the direct effects of HNO on cancer cells, HNO donors may be useful adjuvant agents to cancer chemotherapy. HNO has also been shown to inhibit poly(ADP-ribose) polymerases (PARP) in a breast cancer cell line [80]. As an important component of the DNA repair machinery, PARP is a major target in the design of anticancer agents. Since a number of chemotherapies and radiation therapy are based on inducing DNA damage in cancer cells, inhibition of PARP by HNO donors may increase the efficacy of these treatments.

One attractive attribute of diazeniumdiolates in terms of NO donation is the ability to tune the rate of decomposition based on amine identity [81]. In addition, diazeniumdiolates can be tuned to function as HNO donors, by utilizing a primary rather than secondary amine as the scaffold [82–85]. Recently, we prepared two new diazeniumdiolate–NSAID adducts (NONO-NSAIDs) by derivatizing both a primary and a secondary amine-based diazeniumdiolate with aspirin to produce O^2-(acetylsalicyloyloxymethyl)-1-(*N*-isopropylamino)-diazen-1-ium-1,2-diolate (IPA/NO-aspirin) and O^2-(acetylsalicyloyloxymethyl)-1-(*N*,*N*-diethylamino)-diazen-1-ium-1,2-diolate (DEA/NO-aspirin) [26]. A comparison of their chemical and biological properties demonstrated the expected retention of the analgesic and anti-inflammatory properties of aspirin as well as known effects of HNO or NO donors. In addition, these conjugates showed enhanced cytotoxicity toward human lung epithelial carcinoma cells (A549) while not being appreciably toxic toward human umbilical vascular endothelial cells (HUVECs). Here, we sought to further elucidate the mechanism behind this observed selectivity, with breast cancer as the model.

Materials and methods

Reagents

Unless otherwise noted, chemicals were purchased from Sigma-Aldrich and used without further purification. IPA/NO, DEA/NO, IPA/NO-aspirin, and DEA/NO-aspirin were synthesized and purified according to previously published procedures [26,86]. Stock solutions of NONO-aspirin prodrugs (100–1000×) were prepared in DMSO and stored at −20 °C. The final concentration in media or calcium- and magnesium-free Dulbecco's phosphate-buffered saline (PBS) was adjusted to 0.1% DMSO. Stock solutions of IPA/NO or DEA/NO (1000×) were prepared similarly but in 10 mM NaOH. Concentrations were determined directly prior to use from the extinction coefficient at 250 nm (ε of 8000 M^{-1} cm^{-1}) [87].

Instrumentation

UV-visible spectroscopy was performed with an Agilent Hewlett-Packard 8453 diode-array spectrophotometer equipped with an Agilent 89090A thermostat. Absorbance and fluorescence measurements of 96-well plates were accomplished with a Perkin-Elmer HTS 7000 Bio Assay reader. Cells were counted using a Beckman Coulter Z-2 cell and particle counter. Cell viability and angiogenesis were monitored using a Nikon eclipse TS-100 inverted microscope. An Eppendorf thermocycler was used to prepare cDNA. Sequence detection was then carried out using 7300 Real-Time PCR System from Applied Biosystems. An iBlot gel transfer device from Invitrogen (Carlsbad, CA) was used for dry transfer in Western blots. DNA damage was assessed using a Comet Assay Electrophoresis System (Trevigen Inc., Gaithersburg, MD).

Cell Culture

Human breast adenocarcinoma cells (MDA-MB-231, MCF-7, and MDA-MB-468; American Type Culture Collection, Manassas, VA) were grown as monolayers in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Hyclone, Thermo Fisher Scientific