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Design and discovery of novel quinazolinedione-based redox modulators as therapies for pancreatic cancer

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ABSTRACT

Background: Altered cellular bioenergetics and oxidative stress are emerging hallmarks of most cancers including pancreatic cancer. Elevated levels of intrinsic reactive oxygen species (ROS) in tumors make them more susceptible to exogenously induced oxidative stress. Excessive oxidative insults overwhelm their adaptive antioxidant capacity and trigger ROS-mediated cell death. Recently, we have discovered a novel class of quinazolinediones that exert their cytotoxic effects by modulating ROS-mediated signaling.

Methods: Cytotoxic potential was determined by colorimetric and colony formation assays. An XF24 Extracellular Flux Analyzer, and colorimetric and fluorescent techniques were used to assess the bioenergetics and oxidative stress effects, respectively. Mechanism was determined by Western blots.

Results: Compound **3a** (6-[(2-acetylphenyl)amino]quinazoline-5,8-dione) was identified through a medium throughput screen of ~1000 highly diverse in-house compounds and chemotherapeutic agents for their ability to alter cellular bioenergetics. Further structural optimizations led to the discovery of a more potent analog, **3b** (6-[(3-acetylphenyl)amino]quinazoline-5,8-dione) that displayed anti-proliferative activities in low micromolar range in both drug-sensitive and drug-resistant cancer cells. Treatment with **3b** causes Akt activation resulting in increased cellular oxygen consumption and oxidative stress in pancreatic cancer cells. Moreover, oxidative stress induced by **3b** promoted activation of stress kinases (p38/JNK) resulting in cancer cell death. Treatment with antioxidants was able to reduce cell death confirming ROS-mediated cytotoxicity.

Conclusion: In conclusion, our novel quinazolinediones are promising lead compounds that selectively induce ROS-mediated cell death in cancer cells and warrant further preclinical studies.

General significance: Since **3b** (6-[(3-acetylphenyl)amino]quinazoline-5,8-dione) exerts Akt-dependent ROS-mediated cell death, it might provide potential therapeutic options for chemoresistant and Akt-overexpressing cancers.

1. Introduction

Pancreatic cancer is a multifaceted disorder with poor prognosis. The five-year survival rate is only 5.6% [1]. The major obstacles in the successful treatment of pancreatic cancer are its inherent high resistance to existing chemotherapy and its late-stage detection. Therefore, there is an urgent need to develop new and safe drugs for effective treatment of late stage and resistant pancreatic cancer. A new strategy is to target emerging hallmarks of cancer cells such as metabolic reprogramming and oxidative stress [2]. Aerobic glycolysis, glutamine dependent anaplerosis, de novo lipid and nucleotide biosynthesis and oxidative stress are some of the key bioenergetic alterations observed in cancer cells [3].

Oxidative stress is an important hallmark of most cancer cells including pancreatic cancer [4]. Reactive oxygen species (ROS) including free radicals (superoxide anion, hydroxyl radical) and non-radical species (hydrogen peroxide) are highly reactive and can be detrimental

Abbreviations: CMFDA, 5-chloromethylfluorescein diacetate; DMEM, Dulbecco's Modified Eagle Medium; DMSO, dimethyl sulfoxide; DNA, deoxyribonucleic acid; DPBS, Dulbecco's Phosphate Buffered Saline; DRAQ5®, 1,5-bis{[2-(di-methylamino) ethyl] amino}-4,8-dihydroxyanthracene-9,10-dione; EDTA, ethylene diaminetetraacetic acid; FoxO3a, forkhead box transcription factor 3a; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSH, glutathione (reduced); JNK, c-Jun N-terminal kinase; MnSOD, manganese superoxide dismutase, MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, *N*-acetylcysteine; NADPH, nicotinamide adenine dinucleotide phosphate (reduced); NO-ASA, 3-Nitrooxyphenyl acetylsalicylate; NOX1, NADPH oxidase; NQO1, NAD(P)H dehydrogenase (quinone) 1; OCR, oxygen consumption rate; RIPA buffer, Radio-Immunoprecipitation Assay buffer; ROS, reactive oxygen species; RPMI-1640, Roswell Park Memorial Institute-1640

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when present at a high concentration. Cells maintain a state of redox homeostasis by regulating the equilibrium between ROS generation and scavenging. ROS are generated in biological systems by various enzymatic (i.e. NADPH oxidase) and non-enzymatic (mitochondrial electron transport chain) processes. To maintain ROS below their detrimental levels, several antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, peroxiredoxins, glutaredoxin, thioredoxin and catalase act as ROS scavengers [5].

ROS play an important role as second messengers in cell signaling and regulate a myriad of signal transduction pathways and cell cycle events [6,7]. ROS have also been implicated in various diseases including cancer, neurodegenerative diseases, diabetes, cardiac disorders and mitochondrial diseases. Therefore, maintaining a balance between ROS generation and degradation is essential for normal cell proliferation, growth and survival [8]. Increased synthesis and/or decreased elimination of ROS result in oxidative stress leading to deleterious effects on cellular proteins, lipids and nucleic acids. Moreover, ROS regulate several processes associated with cancer development and tumor invasiveness [9,10]. Increased ROS levels are known to facilitate tumor initiation and progression [11] by inducing DNA damage leading to oncogenic transformation [12]. Therefore, several therapeutic strategies focus on the use of antioxidants as cancer preventive agents [13].

In addition to their tumor promoting actions, ROS exert a critical effect on cell migration by activating specific genes to promote epithelial–mesenchymal like transition and metastasis. Furthermore, cancer cells are known to develop adaptive responses to increased ROS levels to cope with the hazardous effects of oxidative stress [14,15]. These include the activation of redox sensitive transcription factors that increase the expression of endogenous antioxidants, promote survival pathways, induce chemoresistance and alter caspase activation [16,17]. Despite this adaptive mechanism, higher basal ROS levels make cancer cells more susceptible to exogenously induced ROS-mediated cell death. Excessive supply of exogenous oxidative insults will overwhelm the adaptive capacity of cancer cells and promote cell death [16]. Increasing ROS production or decreasing ROS scavenging has shown a therapeutic potential for selectively targeting tumor cells that are under persistent oxidative stress (Fig. 1). This suggests a "two-faced" or dual role of ROS in cancer [18,19]. At lower levels they act as tumor promoters, whereas in excessive amount they cause cell death. Furthermore, ROS-mediated anticancer therapy aids in overcoming resistance associated with other antineoplastic agents [20].

Developing compounds that exploit the high basal ROS levels uniquely present in cancer cells is an innovative and promising approach in drug discovery [16]. ROS inducers, agents decreasing cellular oxidative-buffering capacity, or a combination of both, can produce exogenous oxidative stress. Normal cells have much lower ROS levels as compared to cancer cells, and enjoy a higher antioxidant and oxidative-buffering reserve to deal with exogenous ROS insults. Therefore, it has been shown that these agents are not significantly toxic to normal cells [21].

Since increased bioenergetic alterations and oxidative stress are hallmarks of most forms of cancer, we were interested in discovering compounds that influence cancer cell bioenergetics. The novel compounds described herein targeting the altered cellular bioenergetic characteristic of cancer cells, were identified in a medium throughput screen using an XF24 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MD). XF24 measures the rates of cellular oxygen consumption and extracellular acidification in real time. Cancer cells were exposed to a library of small molecule compounds including novel anticancer agents in our laboratory, known chemotherapeutic agents and novel small molecule compounds designed by us. The screen was performed for finding agents that would alter cellular bioenergetics in cancer cells. Through our screening we identified two classes of potential anticancer agents, triphenylphosphoniums [22] and quinazoline-5,8-diones (**3a**-**f**,**4**) (Table 1). Triphenylphosphoniums decreased cellular oxygen consumption rate (OCR) whereas our novel quinazoline-5,8-diones resulted in tremendous increase in cellular OCR. Our novel small molecule quinazoline-5,8-diones exert the highest level of reported increase in cellular oxygen consumption rate in cancer cells resulting in ROS production and exogenous oxidative stress. The incremental ROS generation induced by these compounds in combination with the higher ROS level present in cancer cells leads to the activation of stress related MAP kinases (p38 and JNK) culminating in cell death.

2. Materials and methods

2.1. Cell culture

Pancreatic cancer (MIA PaCa-2, PANC-1 and BxPC-3), breast cancer (T-47D, MDA-MB-231, MDA-MB-435 and MCF7), lung cancer (NCI-H460 and NCI-H1299), and prostate cancer (PC-3) cell lines were purchased from the American Type Cell Culture (Manassas, VA). HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells were kindly provided by Dr. Bert Vogelstein (The Sidney Kimmel Comprehensive Cancer Center, Baltimore, MD). Human ovarian carcinoma cell line (HEY) naturally resistant to cisplatin (CDDP) was kindly provided by Dr. Louis Dubeau (USC Norris Cancer Center, Los Angeles, CA) [23,24]. OVCAR-8 (ovarian cancer) and multi-drug resistant NCI/ADR-RES cells were obtained from the Developmental Therapeutics Program, NCI (Bethesda, MD). Dr. Carla Grandori (Fred Hutchinson Cancer Research Center, Seattle, WA) kindly provided

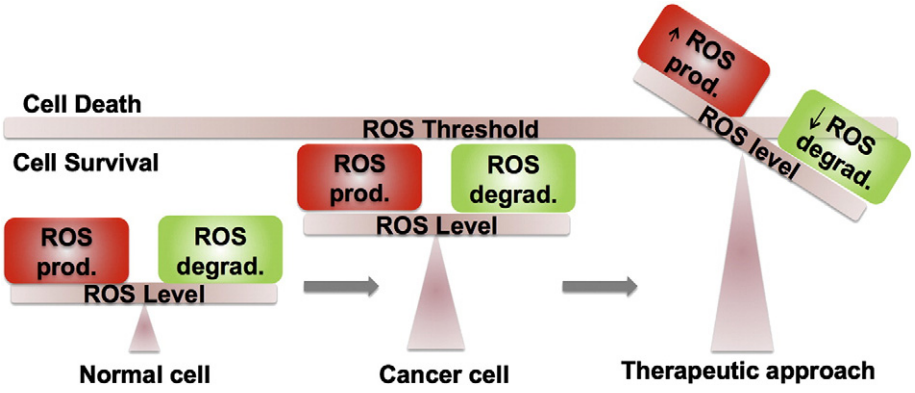

Fig. 1. Rationale for ROS-mediated cancer therapy. Normal cells regulate cellular redox homeostasis by maintaining a balance between ROS generation and scavenging. Oxidative stress is a characteristic of most forms of cancer. Increased ROS levels promote tumor initiation, progression and metastasis. Cancer cells are known to develop adaptive responses to increased ROS levels to protect themselves from the hazardous effects of oxidative stress. However, higher basal ROS levels make cancer cells more susceptible to exogenously induced ROS-mediated cell death. Excessive supply of exogenous oxidative insults will overwhelm the adaptive capacity of cancer cells, promote cell death and can be exploited as a therapeutic approach. (prod., production; degrad., degradation).