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Dual Roles of Oxidative Stress in Metal Carcinogenesis

Jie Xu^a, James T.F. Wise^b, Lei Wang^c, Kortney Schumann^d, Zhuo Zhang^c, and Xianglin Shi^{c,d,*}

^aDepartment of Anesthesiology, Beijing Chao Yang Hospital, Capital Medical University, No. 8 Gongren Tiyuchang Nanlu, Chaoyang District, Beijing 100020, China

^bDivision of Nutritional Sciences, Department of Pharmacology and Nutritional Sciences, College of Medicine, University of Kentucky, Lexington, KY 40536, USA

^cCenter for Research on Environmental Disease, College of Medicine, University of Kentucky, Lexington, KY 40536, USA

^dDepartment of Toxicology and Cancer Biology, College of Medicine, University of Kentucky, Lexington, KY 40536, USA

Abstract

It has been well established that environmental and occupational exposure to heavy metal causes cancer in several organs. Although the exact mechanism of heavy metal carcinogenesis remains elusive, metal-generated reactive oxygen species (ROS) are essential. ROS can play two roles in metal carcinogenesis; two stages in the process of metal carcinogenesis differ in the amounts of ROS activating a dual redox-mediated mechanism. In the early stage of metal carcinogenesis, ROS acts in an oncogenic role. However, in the late stage of metal carcinogenesis, ROS plays an antioncogenic role. Similarly, NF-E2-related factor 2 (Nrf2) also has two different roles, which makes it a key molecule for separating metal carcinogenesis into two different stages. In the early stage, inducible Nrf2 fights against elevated ROS to decrease cell transformation by its antioxidant protection property. In the late stage, constitutively activated Nrf2 manipulates reduced ROS to perform a comfortable environment for apoptosis resistance through an oncogenic role. Interestingly, a cunning carcinogenic mechanism takes advantage of the dual role of Nrf2 to implement the dual role of ROS through a series of redox adaption mechanisms. In this review, we discuss the paradox in the rationales behind the two opposite ROS roles and focus on their potential pharmacological application. The dual role of ROS represents a ‘double-edged sword’ with many possible novel ROS-mediated strategies in cancer therapy in metal carcinogenesis.

Keywords

metal; NF-E2-related factor 2 (Nrf2); reactive oxygen species (ROS); oxidative stress; redox adaptation; carcinogenesis; tumorigenesis

* Address all correspondence to: X. Shi, Center for Research on Environmental Disease, 1095 V.A. Dr., 306 Health Sciences Research Building, Lexington, KY 40536, USA; Tel.: 859-257-4054; Fax: 859-323-1059, xshi5@uky.edu.

I. INTRODUCTION

Metals are one of the leading pollutants of surface and ground waters. Given the development of industries (e.g., metal plating facilities, mining operations, tanneries, etc.), metals in wastewaters continue to be directly or indirectly discharged into the environment. Unlike most organic contaminants, metals are not biodegradable and tend to bioaccumulate or to be biomagnified in living organisms.^{1–4}

Many heavy metals such as arsenic, cadmium, chromium(VI), and nickel are known human carcinogens.⁵ The most common sites for metal-related cancers to develop are the lung and skin. The presence of metals including arsenic, cadmium, chromium, and nickel in cigarettes has led to speculation that these metals contribute to the five-fold increase in lung cancer risk for smokers versus nonsmokers.⁶ Increased risks for cancers of the prostate, kidney, bladder, and liver, as well as for lymphoma and leukemia, have also been reported.⁷

Although the exact molecular mechanisms are not completely understood, the potential of metals to generate reactive oxygen species (ROS) and thus to alter cellular redox states is considered the most important mechanism in metal-induced carcinogenesis.^{8–12} While the balance between cellular antioxidant defense and ROS generation is maintained under normal metabolic conditions, excessive ROS generation can cause oxidative stress.¹³ According to multiple reports, two stages are involved in the process of metal carcinogenesis: an early stage and a late stage. In the early stage of metal carcinogenesis or cell transformation, high levels of ROS are oncogenic and cause DNA damage, inhibition of DNA repair, and alterations of normal signal transduction, ultimately leading to malignant transformation.^{14–18} During the second stage of metal carcinogenesis, metal-transformed cells with low levels of ROS are carcinogenic through the promotion of apoptosis resistance. The metal-transformed cells have an autophagy deficiency resulting in accumulation of p62 and constitutive activation of NF-E2-related factor 2 (Nrf2), which leads to higher levels of antioxidants, decreased levels of ROS, increased levels of B cell lymphoma 2 (Bcl-2), inflammation, and angiogenesis.^{14–18}

Why does the process of metal carcinogenesis have two stages? The best explanation is the dual roles of oxidative stress on metal carcinogenesis. During the first step, the high levels of ROS caused by metal exposure cause the normal cell to undergo cellular transformation to become a transformed or cancer cell, in a process caused by the pro-oncogenic roles of ROS. In the next step, the transformed cell makes use of the antioncogenic role of ROS in creating the microenvironment with low levels of ROS, which promotes the ability of the transformed or cancer cell to escape apoptosis.

In terms of the significance in the process of metal carcinogenesis, the second stage is more significant than the first stage. Both autophagy deficiency and apoptosis resistance are vital to metal tumorigenesis and occur in the last stage. Our recent reports have shown that cells malignantly transformed by the carcinogenic metals chromium, cadmium, and arsenic exhibit autophagy deficiency, a constitutive Nrf2 activation, and decreased ROS. These metals likely share a common pathway for the overall mechanism of carcinogenesis.^{14,15,19,20}

Nrf2 is one of the most important molecules in the two stages, especially the late stage. Nrf2 has dual roles; its protection role and oncogenic role act like a mirror to reflect the dual roles of ROS in metal carcinogenesis. Our recent studies suggest that luteolin can exhibit both antioxidant and pro-oxidant roles to defend against metal-induced cancer cells by targeting Nrf2 in two stages.^{20,21} This natural dietary compound could be exploited as a promising therapeutic for metal carcinogenesis with few toxic side effects.

In this review, we discuss the mechanisms of metal carcinogenesis to build a solid theoretical foundation and to propose tools for fighting against metal related cancer by exploring characteristics of the dual roles of ROS. Our focus is also in identifying an initial molecule, like Nrf2, to break the balance of redox adaption. Additionally, this review may aid in developing novel preventive and therapeutic strategies that lead to new drugs for diseases related to environmental and occupational exposures to metals. Lastly, we hope that this review also stimulates, inspires, and broadens our thinking in the field of ROS in cancer biology beyond cancers that are induced by metals.

II. METAL CARCINOGENESIS

While some metals (e.g., zinc, copper, iron, manganese, and magnesium) are necessary for normal functioning of cells and the survival of organisms at appropriate physiological levels, other metals can be harmful at high concentrations. Exposure to several metals, such as arsenic, cadmium, chromium, and nickel, are carcinogenic.^{1–4} Although the exact molecular mechanism of metal-induced carcinogenesis remains unclear, a vast body of evidence indicates that metal-induced generation of oxidative stress may play a central role in this process.⁹ Epidemiological studies have demonstrated that exposure to these metals causes cancer in animals as well as humans. Chronic exposure to these metals has been linked to skin, lung, gastrointestinal, liver, kidney, and prostate cancers.^{22–28}

The induction of oxidative stress is a major part of the mechanism that explains the mutagenic and carcinogenic effects of these metals. Metal ions for arsenic, chromium, nickel, and vanadium are capable of performing redox reactions in biological systems.⁷ They have been shown to induce the formation of reactive oxygen species *in vivo* and *in vitro* in mammalian cells.²⁹ Typically, mitochondria are the main endogenous source of ROS generation, but metals are also important sources of ROS where highly reactive hydroxyl radical ($\cdot\text{OH}$) is generated via Fenton and/or Haber–Weiss reactions by the breakdown of H_2O_2 .^{7,30–32} Iron and copper are also effective catalysts for Fenton and Fenton-type reactions.³³

Our preliminary studies have shown that exposure of human lung bronchial epithelial (BEAS-2B) cells to arsenic, cadmium, chromium(VI), and nickel results in the generation of a whole spectrum of ROS, including superoxide radical ($\text{O}_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\cdot\text{OH}$), which appear to play a role in malignant transformation.^{15,16,34} However, H_2O_2 itself is not a reactive radical, rather the agent is a precursor to the highly toxic $\cdot\text{OH}$.³⁵ Intracellular biomolecules, such as protein kinases, lipids, and DNA, are potential targets of $\cdot\text{OH}$.^{36,37} Specifically, numerous oxidative DNA adducts have been identified, and their frequency is estimated to be 104 lesions/cell/day in humans.^{36–38} Thus,

oxidative DNA damage induced by $\cdot\text{OH}$ is a key factor influencing the mutagenic and carcinogenic load in organisms living with metals (Fig. 1). Cadmium has been found to generate oxidative stress in a unique way; it has been shown to inhibit antioxidants such as catalase, superoxide dismutase, and glutathione peroxidase (Fig. 1).^{39,40}

DNA repair is an important process in the prevention of mutagenesis. Metal ions can inhibit the repair of DNA damage, and this is thought to be achieved through several mechanisms: interference with base and nucleotide excision repair and interference with the repair of stable DNA adducts.⁴¹ The repair of DNA damage can be inhibited by a diversity of metals, including arsenic, Cd, Co, Cr, and Ni.⁴² Because glutathione (GSH) can reverse this inhibition in many cases, it is possible that metal-induced inhibition of DNA repair might be redox dependent (Fig. 1).⁴³

The mechanisms of metal carcinogenesis may involve multiple pathways to activate redox-sensitive transcription factors, such as growth factor receptors, protein kinases, nuclear transcription factors, and membrane-bound G-protein.^{10,28,44–58} Metals and ROS can act as these signal transduction messengers and/or activators of redox-sensitive signal transduction pathways, affecting the expression of genes that regulate a variety of cellular events.⁴⁴ In turn, this results in the expression of proteins that control inflammation, cellular transformation, tumor cell survival, and cancer cell proliferation, invasion, angiogenesis, and metastasis.⁵⁹ Apparently, an indivisible network contributes to metal carcinogenesis in cooperative ways (Fig. 1).

III. DUAL ROLES OF ROS IN NORMAL CELLS, CANCER CELLS, AND METAL-INDUCED CANCER CELLS

A. The Relationship between ROS and Oxidative Stress

Generally, the balance between cellular antioxidant defense and ROS generation is maintained under normal metabolic conditions. However, exposure due to a xenobiotic (i.e., heavy metals) can lead to excessive ROS generation, which can cause oxidative stress and subsequent damage to lipids, proteins, and DNA. Oxidative stress has been linked to numerous pathologies, particularly cancer. Conversely, reduced oxidative stress can also act as signaling molecules in the maintenance of antioxidant and antiapoptotic conditions, a process termed apoptosis resistance.⁶⁰ In terms of metal carcinogenesis, oxidative stress can directly or indirectly be involved in cell transformation, autophagy dysfunction, apoptosis resistance, and angiogenesis activation, all of which are documented contributors to metal-induced carcinogenesis.¹⁷

B. Physiological and Pathological Function of ROS in Normal Cells

Reactive oxygen and nitrogen species (RNS) are constantly generated in the body from internal metabolism and external xenobiotic exposures.^{61,62} Depending on their concentration, ROS can be beneficial or harmful to cells and tissues. In normal cells, ROS are produced in a controlled manner and sometimes serve useful purposes. ROS that are formed in response to physiological cues act as important signaling molecules to regulate such processes as cell growth and differentiation, inflammation, immune function,

autophagy, and stress responses.^{62–64} Uncontrolled production of oxidants results in oxidative stress that impairs cellular functions and contributes to irreversible oxidative damage, leading to the development of cancer, chronic disease, and toxicity.^{61,65–67} From prokaryotes to humans, ROS function as important regulators of both physiological and pathophysiological outcomes.

Three of the most common and biologically important ROS are $O_2^{\bullet-}$ (superoxide anions), H_2O_2 (hydrogen peroxide), and $\bullet OH$ (hydroxyl radicals); among them, the hydroxyl radical is invariably toxic.⁶⁸ In general, the mitochondria is a major intracellular source of ROS.⁶² However, in the presence of metal ions, highly reactive $\bullet OH$ is generated via Fenton and/or Haber–Weiss reactions. In addition to the generation of $\bullet OH$, the decline of the ROS-scavenging system also leads to the presence of excessive ROS. Both inflict significant damage on cellular proteins, lipids, and DNA by highly reactive $\bullet OH$.^{36,37}

Notably, antioxidant defense mechanisms have the ability to protect cells from oxidative injury, including enzymatic and nonenzymatic systems. Antioxidant enzymes include catalase, superoxide dismutase (SOD), glutathione peroxidase (GPx), and peroxiredoxin III (PrxIII).⁶⁹ The nonenzymatic systems includes vitamins C, E, and B2, coenzyme Q10, glutathione, and β -carotene.⁷⁰ These ROS-scavenging systems are required to maintain cellular redox balance: when the amount of ROS exceeds the capacity of the antioxidant machinery and ROS are not detoxified by those cellular antioxidative agents, oxidative stress occurs.⁷¹ Certainly, it is contradictory that antioxidant defense mechanisms are activated excessively by ROS-scavenging system, resulting in a lower oxidative stress environment to welcome tumor cell survival and proliferation. Therefore, maintaining ROS homeostasis at physiological level is crucial for normal cell growth and survival.

C. The ‘Double-Edged Sword’ Property of ROS is Common in Cancer Cells

Increased ROS is usually accompanied with oncogene activation in the initial steps of malignant transformation. Although the causative relationship of increased ROS and oncogene activation remains unclear, oxidative DNA damage has long been thought to play a central role in carcinogenesis and malignant transformation. However, oxidative DNA damage may be necessary, but not sufficient, for cancer development.⁷² In addition to ROS-induced genomic instability, the increased ROS could mediate various signaling cascades relating to survival, proliferation, resistance to apoptosis, angiogenesis and metastases in pretransformed cells as well as transformed cells, which may promote cancer initiation and progression.^{72–76}

Mounting evidence suggests that ROS are responsible for triggering cell death and reversing drug resistance in tumors. Although treating ROS-inducing tumors with antioxidants is reasonable, paradoxically, the mechanisms underlying many chemotherapeutic agents and ionizing radiation exerted on tumor cells to kill them is not associated with the increase of antioxidants, but rather the production of more ROS leading to irreversible oxidative stress.^{77–79} Such oxidative stress is not often viewed as an adverse event, because although excessive levels of ROS stress can also be toxic to the cells, cancer cells with increased oxidative stress are likely to be more vulnerable to damage by further ROS insults induced by exogenous agents.⁸⁰ Evidence suggests that, compared with their normal counterparts,

cancer cells exhibit greater ROS stress than normal cells do, in part due to oncogenic stimulation, increased metabolic activity, and mitochondrial malfunction.^{81–83} Therefore, manipulating ROS levels by redox modulation is a way to selectively kill cancer cells without causing significant toxicity to normal cells.⁸⁴ The ‘double-edged sword’ property of ROS has been proposed for cancer treatment using both prooxidant and antioxidant roles.^{78,85,86} However, due to the particular complexity of the role of ROS in distinguishing cancer cells at different stages, both oxidant-elevating and oxidant-depleting strategies show complicated advantages and disadvantages. These contradictory statements imply that cancer cells die by the same mechanism that facilitates their survival. This paradox is the most reasonable theoretical foundation on the dual roles of oxidative stress in metal carcinogenesis (Fig. 2).

D. The Redox Adaptation in Metal-Induced Cancer Cells

Due to the critical role of ROS in initiating or promoting the malignant phenotypes of cancer cells, targeting ROS with antioxidants has long been an accepted therapeutic approach.⁷⁹ Dietary antioxidants such as red wine and green tea polyphenols have long been recommended for cancer prevention.^{87,88} Although preventive strategies have been applied in the first stage of metal carcinogenesis, there remains a strong contradiction regarding the application of antioxidants in the late stage of metal carcinogenesis. Of primary concern are the potentially deleterious effects of antioxidant supplements on ROS levels, especially when precise modulation of ROS levels are needed to allow normal cell function or to promote apoptotic cell death of precancerous or pretransformed cells. Moreover, antioxidants interfere with radiotherapy or chemotherapy, which is largely dependent on ROS to induce cytotoxicity. Thus, patients should be advised to be cautious about taking dietary antioxidants during cancer chemotherapy.

Notably, redox alterations in metal carcinogenesis are very complex because of the multiple factors that are involved in the redox regulation and oxidative stress response. Under persistent intrinsic oxidative stress, the malignant cells become well adapted to such stress and develop an enhanced endogenous antioxidant capacity, which makes the malignant cells resistant to exogenous stress.^{89–91} The metal-induced cancer cells that survive intrinsic oxidative stress may have mobilized a set of adaptive mechanisms that not only activate ROS-scavenging systems to cope with the stress but also inhibit apoptosis by upgraded Bcl-2. Therefore, it appears that abrogation of redox adaptation is positively related to effectively therapy strategies for metal carcinogenesis.

Our preliminary studies have also shown that after transformation, metal-transformed cells exhibit constitutive expression of Nrf2 and increased activity of certain ROS-scavenging enzymes and expression of antioxidant proteins, giving rise to a decreased level of constitutive ROS. Seemingly, these findings suggest that the reaction of an adaptive response to oxidative stress should become an essential actor on metal tumorigenesis in late stage. The cunning transformed cell promotes antioxidant adaptive mechanisms to minimize oxidative damage. Redox adaptation by activating ROS-scavenging enzymes to neutralize increased ROS generation seems to be a major mechanism by which cancer cells maintain redox homeostasis. Presumably, abolishing this adaptation mechanism could be an attractive

strategy to specifically target cancer cells and may therefore have significant therapeutic implications (Fig. 2).

IV. DIFFERENT AMOUNT OF ROS IN DIFFERENT STAGES

Intriguingly, for metal carcinogenesis, the dual roles of ROS are dependent on the different amount of ROS in the different stages. Elevated or reduced ROS has a single role. For instance, ROS has an oncogenic role at higher levels in the early stage and an antioncogenic role at lower levels in the late stage, respectively. Different amounts of ROS are associated with different end points in metal carcinogenesis: (1) during moderate enhancement of ROS caused by metal exposures, the normal cell is associated with malignant transformation and disruption of redox homeostasis; (2) metal-transformed cells have lower levels of ROS, which enables them to survive with intrinsic oxidative stress by mobilizing a set of adaptive mechanisms, which not only activates ROS-scavenging systems to cope with the oxidative stress but also inhibits apoptosis.

For metal carcinogenesis, we can look at controlling the amount of ROS in the different stages in several ways: (1) by activating certain molecular pathways to decrease ROS in nontransformed cells in order to prevent metal-induced malignant transformation; (2) by locating agents that target the key cellular antioxidant system in order to exert a more efficient impact; and (3) by inducing the elevation of the generation of ROS directly, activating the defense against the cell that embraces a low level of ROS. Taken together, the dual role of ROS provides a great opportunity for researchers whose aim is to exploit a 'double-edge sword' to cope with metal carcinogenesis (Fig. 3).

V. THE FORMATION PROCESS OF DUAL ROLES OF ROS IN METAL CARCINOGENESIS

A. Two Stages in Metal Carcinogenesis

To discuss the topic of the dual role of oxidative stress in metal carcinogenesis, we must first understand the two stages in metal carcinogenesis and the close relationship between the dual role of ROS and the two stages in metal carcinogenesis. ROS plays a major role in metal-induced malignant transformation, which is also known as the early stage. The role of ROS in this stage indicates that it can be used a tool for prevention of metal-induced malignant transformation and carcinogenesis. We have previously shown that inhibition of reactive oxygen species (ROS) by specific antioxidants such as SOD (against $O_2^{\bullet-}$) and catalase (against H_2O_2) inhibited Cr(VI)-induced malignant cell transformation.¹⁶ Our studies have also shown that inhibition of ROS decreases arsenic-transformed cell survival.⁹² These results indicate that ROS are generated in response to metal exposure and play a major role in metal-induced malignant transformation. Thereby, we can conclude that elevated ROS is positively related to metal carcinogenesis, which includes an oncogenic role of ROS.

Decreased ROS generation triggers a reaction to establish an advantageous circumstance against oxidative stress in metal-transformed cells, termed as the late stage. It is very interesting that the basal ROS levels in the transformed cells are lower than their passaged

match control counter parts. Our studies have shown that transformed cells exhibit a decreased capability of ROS generation.⁹³ This decreased production of ROS contributes to some of the malignant features, such as increased proliferation and apoptosis resistance.³⁴ Low basal ROS levels show an increased survival of transformed cells. The decreased production of ROS could be the result of an increased expression of antioxidant enzymes. In arsenic-, cadmium-, and Cr(VI)-transformed cells, the levels of antioxidants are higher than those in the passaged match nontransformed cells, which contributes to decreased ROS generation and increased apoptosis resistance.⁹³ The inhibition of catalase could increase ROS levels and rescue the apoptosis capability of these cells.⁹⁴ Thus, we can conclude that reduced ROS is positively correlated to metal tumorigenesis, supporting an antioncogenic role of ROS. Taken together, it appears that the dual role of ROS depends on different redox-mediated mechanisms in two stages. The late stage is more important than the early stage because the mechanism of Nrf2 signaling not only activates the ROS-scavenging systems but also inhibits apoptosis by upregulating Bcl-2.^{14–16,19,34} Future clinical research must aim toward overcoming drug resistance in the second stage (Fig. 4).

B. Nrf2 is the Most Important Molecular for Dual Roles of Oxidative Stress in Metal Carcinogenesis

Nuclear factor (erythroid-derived2)-like2, also known as NFE2L2 or Nrf2, is a basic region-leucine zipper-type transcription factor that is encoded by the NFE2L2 gene in humans.⁵¹ Nrf2 is the guardian protein of redox homeostasis. Under oxidative stress conditions, it activates a series of antioxidant and cytoprotective genes that contain a cis-acting enhancer sequence, termed the antioxidant response element (ARE).^{95,96} These protective enzymes are involved in the detoxification of ROS and xenobiotic compounds.⁹⁷ A major emerging function of Nrf2 from studies over the past decade is its role in resistance to oxidative stress. The protective nature of Nrf2 could also be appropriated by cancer cells to create a prosurvival microenvironment for tumor growth and drug resistance.⁹⁸ Nrf2 is suppressed under a basal condition through Keap1 (Kelch-like erythroid cell-derived protein with CNC homology-associated protein 1)-dependent ubiquitination–proteasomal degradation and is activated by oxidants and electrophiles via modification of critical cysteine thiols of Keap1 and Nrf2.⁹⁹ The Nrf2–Keap1 module is of primary importance in maintaining the homeostatic milieu. Knockout of Keap1 in mice resulted in constitutive activation of Nrf2 from stabilization of Nrf2 protein.¹⁰⁰

The impact of Nrf2 on cancer is complex. An emerging concept is that Nrf2 acts as a ‘double-edged sword.’ On one hand, Nrf2 is required for protecting the body against cancer through endogenous and pharmacological anticancer agents and on the other, it is constitutively activated in some tumors, resulting in a pro-survival phenotype to promote tumor growth and resistance to oxidants and anticancer drugs.^{98,99,101} The potentially more harmful than beneficial effects of Nrf2 on cancer were gleaned from the observations that some tumors have persistently elevated expression of ARE containing genes, indicating that cancer cells hijack the protective power of Nrf2 to increase resistance to oxidants and cancer killing agents.

Activation of Nrf2 in tumors is unlikely to be a cancer-initiating event but rather a result of selection during tumor development; Keap1 knockout mice with elevated Nrf2 functions did not spontaneously develop cancer over a 2-year period.¹⁰² These findings suggest that specific inhibitors of Nrf2 could be developed for therapy against cancers that have elevated Nrf2 activity.¹⁰¹

C. The Dual Roles of Nrf2 in the Two Stages

Regarding the first stage, our previous studies showed that exposure of human lung bronchial epithelial (BEAS-2B) cells to Cr(VI) generated a whole spectrum of ROS, which aggravate Cr(VI)-induced cell transformation.^{16,44} Inhibition of ROS is an important approach to protecting cells from Cr(VI)-induced transformation. Notably, malignant cells, which are characterized by their autonomy, are very different from dysplastic (not completely neoplastic) cells in a premalignant lesion. Premalignant cells are under much greater control from inflammatory cells and other stromal cells in their microenvironment. Moreover, they have not yet reached a level of DNA damage that makes them malignant. Therefore, enhancement of Nrf2 activity, which would amplify antioxidants, neutralize further oxidative or mutagenic stress, and restore cellular redox balance, appears to be beneficial during premalignant states.^{103–105} As such, the biological time context is important: Nrf2 activity is desirable (for the host organism) in early stages of metal carcinogenesis because the host is seeking to suppress premalignant carcinogenesis. A variety of studies show that the activation of inducible Nrf2 is anticarcinogenic in the early stage of carcinogenesis (from normal cells to malignantly transformed cells) through the upregulation of its target antioxidants to reduce the levels of ROS.^{104,106–108} In our recent study, we show that activation of inducible Nrf2 by certain natural compounds can not only decrease the amount of ROS but also acts as protector against Cr(VI)-induced malignant cell transformation of BEAS-2B cells.²⁰

While in the early stage Nrf2 is inducible and play a protective role, Nrf2 becomes undesirable in the late stage of metal carcinogenesis. This occurrence is due to the ability of constitutively activated Nrf2 to ultimately playing an oncogenic role and cause malignant cancer cells resistant to apoptotic cell death and/or treatments.¹⁰⁴ In Cr(VI)-transformed cells, ROS may function as an inhibitor of tumorigenesis by the induction of apoptosis. In contrast, constitutive expression of Nrf2 is oncogenic through a protection of cancer cells from oxidative stress and chemotherapeutic agents in the late stages of carcinogenesis (from transformed or cancer cells to tumor).^{16,104,105} Constitutive expression of Nrf2 is found in several types of human cancer cell lines and tumors.^{109–111} Therefore, the stage of carcinogenesis determines whether Nrf2 acts as a cancer preventive or oncogenic agent.^{103,105} In addition, constitutively activated Nrf2 in cancer cells binds to ARE sites of Bcl-2 and Bcl-XL gene promoter, leading to up-regulation of Bcl-2 and Bcl-XL and development of resistance to apoptosis.¹¹² In our recent studies, we showed that in arsenic-, Cd-, Cr(VI)-, and Ni-transformed cells, Nrf2 is constitutively activated, and this activation upregulates antioxidant and antiapoptotic proteins through the binding of Nrf2 to the ARE sites of these target proteins.^{14,15,113,114} The outcome is decreased levels of ROS and the development of apoptosis resistance, an environment favorable for progression of transformed cells and tumor formation. When Nrf2 becomes constitutively activated, its inducible nature is lost in

metal-transformed cells. Notably, metal-induced ROS cause only an intermittent and variable effect on Nrf2 in the early stage, whereas the activation of Nrf2 through alterations is continuous and invariable in the late stage (Fig. 5).

D. Nrf2 Activation Underlying Two Mechanisms: Keap1-Dependent and P62-Dependent

By understanding the dual roles of Nrf2 in two stages, we conclude that Nrf2 activation originates from two different mechanisms: Keap1-dependent and p62-dependent mechanisms. Subsequently, we expect to screen a promising agent that activates Nrf2 by stabilizing the protein of Nrf2 via targeting Keap1, which is dynamically regulated and provides intermittent increase in Nrf2 (neither constitutive nor prolonged). This Keap1-dependent mechanism of Nrf2 activation is antioncogenic and is typical Nrf2 activation induced by natural cancer preventive compounds in the early stage (canonical mechanism).^{115,116} This mechanism is different from the p62-dependent one, which is a noncanonical mechanism, in which Nrf2 loses its inducible nature and expression of this protein is not transient but constitutive and prolonged.¹¹⁷ This p62-dependent mechanism of constitutive Nrf2 activation is oncogenic in the late stage.^{111,115,116,118}

E. The Canonical Mechanism Underlying the Dual Roles of Nrf2 in Arsenic-Induced Carcinogenesis

Arsenic(III)-induced ROS have been shown to have oncogenic properties in the premalignant stage. Exposure of human lung bronchial epithelial cells to arsenic generates ROS, which are responsible for malignant transformation of these cells.⁹³ During this stage, the Nrf2 is inducible and regulated by Keap1. We demonstrated that arsenic-induced cell transformation was further enhanced by knockdown of Nrf2 by shRNA transfection after 2 or 3 months of exposure. By upregulating antioxidant enzymes to reduce ROS, the increased presence of Nrf2 has an antioncogenic property within the cell.⁹³

The protective function of Nrf2 in the cells against cell transformation might be lost when the cells are persistently exposed to arsenic. Furthermore, the protective role of Nrf2 in cell transformation is reversed after four months of arsenic exposure as its inducible property is lost. At this stage, the constitutive overexpression of Nrf2 further upregulates antioxidant enzymes and antiapoptotic proteins in the transformed cells. The phenomenon causes low cellular ROS levels and the acquisition of apoptosis resistance. A low level of ROS increases survival and proliferation of transformed cells and tumorigenesis.³⁴ In this stage, constitutive overexpression of Nrf2 has an oncogenic property. This protein is mainly regulated by p62 rather than Keap1. Furthermore, high expression of p62 due to defective autophagy leads to accelerated Nrf2 activation, which results in forming a positive feedback loop. These results suggest that Nrf2 has an antioncogenic or oncogenic role, depending on the stage of cell transformation.¹⁴ Our findings provide a potential chemoprevention and chemotherapy strategy for metal-induced carcinogenesis through the manipulation of Nrf2 expression and activity.

F. Nrf2 Impact on Inflammation in Metal Carcinogenesis

Our results indicate that before transformation, ROS plays a critical role, while Nrf2 has no role in Cr(VI)-induced inflammation. After transformation, however, a constitutively high

level of Nrf2 is critical in maintaining the inflammation, while ROS has no role.¹¹³ In chromium-transformed cells, the constitutive activation of Nrf2 is responsible for the production of inflammatory regulatory proteins such as cyclooxygenase-2 (COX-2), tumor necrosis factor alpha (TNF- α), and nuclear factor- κ B (NF- κ B).¹¹⁹

Our published and ongoing studies have shown that arsenic-transformed cells exhibit higher levels of COX-2, TNF- α , and NF- κ B in a Nrf2-dependent mechanism, creating a chronic inflammatory microenvironment in these cells.⁹³ The sustained chronic inflammatory microenvironment may contribute to tumor development through several mechanisms, including the release of prostaglandin E2 (PGE2), which can suppress antitumor response and facilitates tumor growth by inducing a range of angiogenic factors.^{120,121}

In summary, Nrf2 is the most important key molecule for dual roles of oxidative stress in metal carcinogenesis. It is possible that without Nrf2 there would be no dual roles of oxidative stress in metal-induced cancers. Moreover, Nrf2 seems like a dividing point, which separates the ROS oncogenic and antioncogenic role as well as the early and late stage by producing an oxidative or antioxidative environment. Overall, abundant evidence shows that targeting Nrf2 can be a safe and effective strategy for the chemoprevention and chemotherapy of metal carcinogenesis. It should be emphasized that drugs cause only an intermittent and variable effect on inducible Nrf2, whereas the activation of constitutive Nrf2 through mutations is constant and invariable.

G. Autophagy Deficiency in Metal-Transformed Cells

Autophagy is a self-degradative process that is important for the disposal of excess or damaged organelles and clearance of aggregation-prone proteins.¹²² Autophagy is essential for housekeeping functions and is induced in a number of stress responses.¹²³ Autophagy is also involved in tumor development, which is thought to play a dual role in carcinogenesis. For example, autophagy has a protective role in for cancer cells under nutrient starvation, radiotherapy, and certain drugs.¹²⁴ Meanwhile, inhibiting autophagy increases cancer development in certain circumstances as it has been reported that impaired autophagy is found in a variety of cancer cells and tumors.^{122,125} These reports suggest that autophagy's role in cancer is cancer context dependent.

Carcinogenic metals are able to induce autophagy in normal cells.^{94,126} The activation of autophagy has been reported cadmium- or arsenic-exposed cells and is likely to be the cell self-defense mechanism against metal-induced oxidative damage.^{15,126} The potency of metal in autophagy induction is different in normal and transformed cells. Metals have a limited ability to induce autophagy in transformed cells. In arsenic-transformed cells, only a limited number of autolysosomes are generated when autophagosomes and lysosomes become fused, suggesting that arsenic transformed cells are autophagy impaired.¹⁴ A similar result was observed in cadmium-transformed cells.¹⁵ These results show that metal-transformed cells have a property of autophagy deficiency. p62 is one of the selective substrates for autophagy; it has been found to accumulate in autophagy-deficient cells.¹²²⁷ p62 increases the levels of Nrf2, creating a positive feedback loop between p62 accumulation and Nrf2 activation in autophagy deficiency. The accumulation of p62/Nrf2 in transformed cells may further contribute to upregulated antioxidant enzymes, antiapoptotic proteins, and

inflammatory factors and angiogenesis proteins, which could play an important role in metal carcinogenesis (Fig. 6).

On the other hand autophagy is a catabolic process that enables cells to break down intracellular organelles (e.g., ribosomes and mitochondria) and allows them to recycle for biosynthesis and energy metabolism.¹²⁸ This supports cell survival mechanisms against a stress environment.¹²⁴ However, autophagy is a type II programmed cell death, which can mediate cellular demise through excessive self-digestion and degradation of essential cellular constituents.¹²⁸ Thus, the cytotoxic effect of autophagy is well recognized in type II cell deaths.

In our experimental setting using arsenic-, cadmium-, and Cr(VI)-transformed cells, autophagy involves a cell death pathway, not a survival function, suggesting that defective autophagy in the transformed cells has a beneficial effect for survival mechanisms. Notably, upregulation of Bcl-2/Bcl-xL may further contribute to dysregulation of autophagy in the cadmium and arsenic transformed cells because Bcl-2/Bcl-xL inhibits the autophagy function through the binding of the BH-3 domain of Beclin-1, which is necessary for the induction of autophagy.^{14,15}

However, nickel is a special case because it induces autophagy enhancement without autophagy deficiency, which is different to arsenic, cadmium, and Cr(VI). The high expression levels of Nrf2 in Ni-transformed cell activate Stat3, which leads to autophagy enhancement. Negative regulation between apoptosis and autophagy in response to Ni stimuli enhances the carcinogenic potential and survival of transformed cells.¹¹⁴ We can speculate that autophagy involves a cell survival pathway and not a death function in a Ni-transformed cell.

H. p62 Promotes the Constitutive Activation of Nrf2 Transcriptional Programs

In autophagy competent cells the p62 protein, known as sequestosome (SQSTM1), binds to both polyubiquitin on the autophagy cargo via its ubiquitin-associated domain and to the autophagosome protein microtubule-associated protein 1A/1B-light chain 3 (LC3) via its LC3-interacting region (LIR) domain, which directs cargo to autophagosomes for degradation.¹²⁹ Thus, when autophagy is induced, p62 localizes to autophagosomes and is subsequently degraded after autophagy is completed.¹²⁹ Under normal conditions, p62 functions as an autophagy substrate.¹²⁷ Conversely, in autophagy deficiency (blockage of the fusion between autophagosome and lysosome) p62 accumulates in the autophagosomes. Our preliminary results show that lysosome-associated membrane protein 2a (LAMP2a), a protein important for the fusion between autophagosomes and lysosomes, was decreased in Cr(VI)-transformed cells.^{130–132} The accumulated p62 competitively binds to the Nrf2-binding site of Keap1, a negative regulator of Nrf2, causing decreased binding of Keap1 to Nrf2 and leading to constitutive activation of Nrf2. This promotes expression of the Nrf2 target gene, including antioxidant enzyme genes. Further, Nrf2 regulates p62 expression through direct binding to the antioxidant response element (ARE) binding motif of the p62 promoter, suggesting a positive feedback of these two proteins.¹³³

The role of p62 in tumorigenesis of arsenic-transformed cells could be determined by tumor sizes under various treatments and control.¹⁴ p62 is able to stimulate Nrf2 activity via a positive feedback loop between p62 and Nrf2.¹³³ This p62-dependent Nrf2 activation, a noncanonical mechanism of constitutive Nrf2 activation, is constitutive and is considered carcinogenic.^{117,118} Collectively, p62 is at the crossroads of oxidative stress, autophagy, apoptosis, and cancer.^{115,134,135} Impaired autophagy leads to accumulation of autophagosomes and increase of p62, promoting tumorigenesis.¹³⁶ This protein is an emerging regulator of tumorigenesis. Modulation of p62 by autophagy is a key factor in tumorigenesis.

I. A Positive Feedback Loop among P62, Nrf2 and Keap1

p62 is the positive upstream regulator of Nrf2 and multiple report have shown that p62 is a substrate of autophagy and that autophagy impairment causes accumulation of this protein.^{127,137} Recent studies have demonstrated that p62 has a critical role in an oxidative stress response pathway by its direct binding to the ubiquitin ligase adaptor Keap1, an inhibitor of Nrf2. The binding between p62 and Keap1 makes Keap1 unavailable to inhibit Nrf2, leading to Nrf2 constitutive activation. Our studies have shown that in cadmium-transformed cells, a high level of p62 is accumulated due to autophagy deficiency, resulting in a decreased level of Keap1 and constitutive activation of Nrf2.¹⁵ It has been reported that Nrf2 is able to bind to the antioxidant response element (ARE) regions of p62 gene. Thus, p62 enhances Nrf2 activity through inactivation of Keap1.¹³⁸ Nrf2, in turn, upregulates p62 expression by binding of Nrf2 to ARE sites on the p62 promoter.^{14,133} Constitutively expressed Nrf2 and p62 form a positive feedback loop. Additional, depletion of p62 by siRNA transfection resulted in a decrease of arsenic(III) and cadmium-induced Nrf2 expression in transformed cells. Knockdown of Nrf2 by siRNA transfection also attenuated arsenic(III)- and cadmium-induced p62 expression in the cells.^{14,15} This loop could increase several downstream functional proteins including antioxidant enzymes, antiapoptotic proteins, inflammatory factors, and angiogenic molecules. All of these characteristic changes within the cell help to establish a favorable low ROS circumstance and enhance metal tumorigenesis. Notably, the comparatively high level of p62 in the transformed cells is not a direct cause of Nrf2 activation because the Nrf2 binding to the ARE regions of the p62 promoter is not higher in the nonstimulated transformed cells than in the normal cells. High expression of p62 seems mainly due to autophagy deficiency in the transformed cells.

J. Apoptosis Resistance

Apoptosis is well described as programmed cell death and serves as a natural barrier to tumor development.¹³⁹ Notably, apoptosis occurs without inducing inflammation, thereby minimizing damage to nearby cells and tissue.¹⁴⁰ The apoptotic machinery is controlled by pro- and antiapoptotic members of the Bcl-2 family.¹³⁹ Cancer cells have a variety of strategies to escape apoptosis, such as the loss of the p53 tumor suppressor and an increase in expression of antiapoptotic proteins like Bcl-2 and Bcl-xL.^{141,142} Acquired resistance to apoptosis is a hallmark of all cancers.¹⁴² Under normal circumstances, apoptosis eliminates DNA-damaged or mutated cells. Apoptosis resistance assists the malignant cell to escape from programmed cell death during carcinogenesis. Disruption of apoptosis plays a major role in tumor formation and malignant progression.¹⁴³

In terms of metal carcinogenesis, especially in the late stage, Nrf2 has been reported to play a critical role in causing apoptosis resistance of metal transformed cells.¹⁵ On one hand, it is well known that ROS is a key mechanism for toxic metal-induced apoptosis.⁵⁹ While constitutive activation of Nrf2 decreases the basal levels of ROS by upregulation of antioxidant enzymes, the enhanced ROS scavenging system could be responsible for apoptosis resistance in transformed cells.³⁴ The apoptosis resistance due to low ROS levels may increase cell proliferation, providing a favorable environment for tumorigenesis of transformed cells. In arsenic-transformed cells, inhibition of catalase could increase ROS level and rescue the apoptosis capability.⁹⁴ Cadmium-transformed cells have apoptosis resistance compared with parental nontransformed cells, which is correlated with low basal ROS levels in transformed cells.¹⁵

On the other hand, Nrf2 is able to bind to the ARE region of Bcl-2 gene, leading to upregulation of Bcl-2 and increased cell survival.^{144,145} Our studies have demonstrated that Nrf2-dependent increased antiapoptotic proteins along with decreased ROS generation might be responsible for apoptosis resistance in arsenic- and cadmium-transformed cells, as well as Nickel-transformed cells.^{14,15,114} Furthermore, apoptosis resistance not only facilitates the metal carcinogenic process but also develops a shelter of low ROS against anticancer agents.¹⁰⁵ Above all, constitutive activation of Nrf2 is the pivotal factor in apoptosis resistance of metal transformed cells. Thus, there seems to be a strong rationale for the development of new Nrf2-inhibitory agents for use in cancers in which genetic mutations cause constitutive activation of the Nrf2 pathway.

K. Autophagy Deficiency Resulted from Constitutive EGFR Activation in Cr(VI)-Transformed Cells

Epidermal growth factor receptor (EGFR) regulates various key cellular processes including proliferation, survival, and tumorigenesis.¹⁴⁶ In 40–80% of non-small cell lung cancer (NSCLC), EGFR was activated.^{147,148} Our previous studies have also shown that EGFR was activated in the lung tissues from Cr(VI)-exposed animals and in lung tumor tissues from a nonsmoking worker occupationally exposed to Cr(VI) for 19 years with a diagnosis of stage 1 squamous lung carcinoma (T1N0M0).¹⁹ Our ongoing studies have found that autophagy deficiency resulted from constitutive EGFR activation in Cr(VI)-transformed cells. Furthermore, this process consists of two steps: (1) initiation of autophagy by a decrease in mammalian target of rapamycin (mTOR), an autophagy inhibitor, with increases in transcription factor EB (TFEB), Beclin-1, and autophagy-related protein 7 (ATG7), and (2) the blockage of the fusion between autophagosomes and lysosomes by inhibition of LAMP2a, which is an important protein for lysosome biogenesis and for the fusion between autophagosomes and lysosomes to generate autolysosomes.^{122,130–132,149} These steps are both indispensable and originate from EGFR activation. Therefore, we hypothesize that in Cr(VI)-transformed cells activation of EGFR inhibits LAMP2a generation, resulting in autophagy deficiency by blocking the fusion between autophagosomes and lysosomes followed by a series of behaviors of carcinogenesis.

Although EGFR activation blocks the fusion between autophagosomes and lysosomes, inhibition of EGFR is not expected to increase the fusion because EGFR also functions as

autophagy initiator and under EGFR inhibition there is no autophagosome generated. The downregulation of LAMP2a by constitutive EGFR activation blocks the fusion between autophagosomes and lysosomes, which provides a linkage between EGFR activation and autophagy deficiency. This new concept is innovative not only in Cr(VI) carcinogenesis but also in a broad sense. This newly recognized function of EGFR broadens our knowledge in the field of EGFR cancer biology, including EGFR-based cancer prevention, and helps move this field forward (Fig. 6).

VI. A 'DOUBLE-EDGED SWORD', LUTEOLIN, DEFENSE AGAINST DUAL ROLES OF ROS IN METAL CARCINOGENESIS

Overall, this review has demonstrated the integration of Nrf2/p62, ROS, autophagy, apoptosis, and cell survival in metal-induced carcinogenesis, which displays the formative process of the dual roles of ROS in metal carcinogenesis. First metal-induced ROS have shown oncogenic properties in the premalignant stage. In our studies, exposure of human lung bronchial epithelial cells to various metals generate ROS, which are responsible for malignant transformation of these cells.^{14–16,134} Then, after transformation (the postmalignant stage), ROS plays an antioncogenic role. A low level of ROS is one of the main reasons for increasing survival of transformed cells and tumorigenesis. High expression of p62 due to defective autophagy leads to constitutive Nrf2 activation. In turn, constitutive Nrf2 was followed by the increase in antiapoptotic proteins (Bcl-2/Bcl-xL), antioxidant enzymes (catalase, SOD1, and SOD2), even upregulation of p62 by a positive feedback loop. The decreased ROS and increased Bcl-2/Bcl-xL levels due to constitutive Nrf2/p62 expression are responsible for the development of apoptosis resistance. This process promotes cell survival, proliferation, and carcinogenesis of transformed cells.^{14,15,19,113} Based on the two roles of ROS presented in our previous studies, the following approach is proposed to find a 'double-edged sword' to prevent metal-induced carcinogenesis. In the first stage of metal carcinogenesis or prior to cell transformation, up-regulation of Nrf2 may reduce metal-induced ROS generation and prevent metal-induced cell transformation. In the second stage of metal carcinogenesis (after cell transformation), downregulation of constitutive Nrf2 expression may reduce antioxidant protein levels and increase ROS generation. Both increased ROS generation and reduced Bcl-2 and Bcl-xL expression will increase apoptosis sensitivity. These steps result in decreased proliferation and survival of transformed cells as well as a decrease in carcinogenesis.

We searched the literature and screened a variety of natural compounds in an attempt to select those with two types of properties: (1) to activate Nrf2 and decrease ROS in normal cells to prevent metal-induced malignant transformation and (2) to inhibit constitutive Nrf2 activation and increase ROS and decrease Bcl-2 in metal-transformed cells to suppress survival of these transformed cells and to prevent tumorigenesis. We found that luteolin, a natural flavonoid compound, has these dual properties.

Cancer prevention with naturally occurring dietary agents has gained immense interest in recent years due to the safety, low toxicity, and general availability of these compounds.¹⁵⁰ Flavonoids are a group of naturally occurring polyphenolic compounds with a wide range of

biological effects.¹⁵¹ Luteolin (3',4', 5,7-tetrahydroxyflavone) is a common dietary flavonoid found in fruits, vegetables, and medicinal herbs with recognized antioxidant, antiinflammatory, and anticancer activities.^{152–155} According to the dual roles of Nrf2 in different stages, luteolin can exhibit both antioxidant and prooxidant activities acted as antioncogenic role in metal carcinogenesis.

We can take our model using the Cr(VI) exposure to human lung bronchial epithelial (BEAS-2B) cells as an example.^{19,20,113,134} In the early stage, luteolin activates inducible Nrf2 in normal BEAS-2B cells, which increases cellular levels of antioxidants and decreases Cr(VI)-generated ROS. The outcome is inhibition of Cr(VI)-induced malignant cell transformation, leading to a protection against the first stage of Cr(VI)-induced carcinogenesis. These activities are cancer preventive or antioncogenic. In the late stage, three key steps contribute to the course in which luteolin interrupts the process of tumorigenesis in Cr(VI)-transformed cells observed in our recent study²⁰: (1) this natural compound reduces constitutive Nrf2 activation; (2) luteolin inhibits the binding between Nrf2 and the promoter regions of the target antioxidant and antiapoptotic proteins; and (3) luteolin decreases the levels of antioxidant and antiapoptotic proteins whose activities are of a concern to the anticancer role of ROS implemented by prooxidative or proapoptosis. These strategies take advantage of the cascade of molecular events that occur over time through the whole carcinogenesis process. These luteolin strategies take advantage of the double role of Nrf2, enabling a moderate promotion of Nrf2 in the early stage of Cr(VI)-induced carcinogenesis and reduction of the constitutive and high level expression of this protein in the later carcinogenesis stage. The results may also be used to develop a similar strategy against other carcinogenic metals such as arsenic, cadmium, and nickel.^{14,15,156–159} These carcinogenic metals share a common mechanism with Cr(VI) and also generate ROS, cause oxidative stress, and induce cancers of various organs. In short, luteolin provides a novel tool in defense against the dual role of ROS with key regulatory proteins (Nrf2) in metal-induced carcinogenesis, which can be used as an effective strategy in chemoprevention and chemotherapy (Fig. 7).

VII. ACQUIRE ENLIGHTENMENT FROM THE DUAL ROLE OF OXIDATIVE STRESS IN METAL CARCINOGENESIS

ROS have a dual role in metal carcinogenesis (as discussed above): They cannot only play an oncogenic role, but they can also act in an antioncogenic role. This poses a great challenge for cancer researchers is determining how to exploit this dual property of ROS for therapeutic development in different stages. We have gained several insights from the dual role of oxidative stress in metal carcinogenesis as followings areas: redox adaptation and drug resistance, role in the two-stage carcinogenesis, combination of ROS-mediated approaches, and the redox adaptation in metal-induced cancer stem cells (Fig. 8).

A. Redox Adaptation and Drug Resistance

Theoretically, two ROS-mediated cancer therapy strategies are possible: (1) inhibition of the ROS “survival signaling” or (2) stimulation of ROS production at high enough levels to trigger death pathways.¹⁵⁸ Cancer cells exhibit greater ROS stress than normal cells do, due

in part to oncogenic stimulation, increased metabolic activity, and mitochondrial malfunctions.^{81–83} Conceivably, these malignant cells would be more dependent on antioxidants for cell survival and therefore more vulnerable to further oxidative insults induced by ROS-generating agents or by compounds that abrogate the key antioxidant systems in cells. Further studies have demonstrated that ROS producing agents could facilitate the therapeutic effects of traditional cancer treatment (in combination with the conventional chemotherapy or radiotherapy).^{86,159} However, simply adding ROS-generating agents may not always lead to the preferential killing of cancer cells.

Redox adaptation through the elevation of endogenous antioxidants and the activation of cell survival pathways may confer an increased capacity to tolerate exogenous stress and insults, defined as a decrease in apoptotic execution, which is a common phenomenon in tumor cells. The increased capacity of redox adaptation in cancer cells has long been linked to drug resistance.¹⁶⁰ The more reductive the cell, the more resistance to the chemotherapeutic agent the cell. In other words, excessive activation of ROS-scavenging system is the best explanation for high chemoresistance.¹⁶⁰

In normal cells, the scavenging of free radicals through the use of antioxidants is an effective means of protecting these cells from ROS-induced malignant transformation. For the transformed cell, antioxidants, or low levels of ROS that induce antioxidant production, appear to contribute to the growth of the tumor cell and enhance the resistance of these cells to anticancer therapies.¹⁶¹ Metal-induced cancer cells exhibit increased ROS generation that may promote cell proliferation and in many cases might be coupled to redox adaptation to promote cell survival. Some cancer cells (especially those in the late stage) have become highly adapted to intrinsic oxidative stress with upregulated antioxidant capacity. This redox adaptation not only enables the cancer cells to survive under increased ROS stress but also provides a mechanism of resistance to many anticancer agents, due to the increased tolerance of exogenous stress and an upregulation of survival molecules.¹⁵⁸

To achieve therapeutic selectivity and overcome drug resistance associated with redox adaptation, it is important to design a strategy that exploits the redox difference between normal cells and cancer cells and that disables the redox adaptation mechanism in cancer cells. One such approach is to target the key redox regulatory mechanisms that control both the level of ROS and the function of redox-sensitive survival proteins. Notably, for the cancer cells that have adapted to oxidative stress by increasing their antioxidant capacity (often by up-regulating Nrf2), the use of simple ROS-generating agents might not be effective.¹⁵⁸ A better strategy might be to use compounds that abrogate the adaptive mechanisms. A caveat is associated with cancer-cell adaptation to intrinsic oxidative stress, however, and one should not expect that exogenous ROS-generating agents would have preferential activity against cancer cells.

As Nrf2 plays a vital survival role in the late stage of metal carcinogenesis, it is logical to argue that increased Nrf2 activity could be tumor promoting by being protective for cancer cells, which might cause chemotherapy resistance by several mechanisms. Constitutive activation of Nrf2 is evident in several human cancer cell lines as well as in tumors.^{110,162,163} The upregulation of antioxidant capacity in adaptation to oxidative stress in

cancer cells can confer with drug resistance by constitutively high overexpression of Nrf2 in metal carcinogenesis. Abrogation of such drug-resistant mechanisms by redox modulation could have significant therapeutic implications. We argue that the unique redox regulatory molecular of cancer cells might be an effective strategy to eliminate these cells. Our studies have demonstrated that luteolin could increase generation of ROS and an altered redox status by targeting constitutively Nrf2 in metal-transformed cells (Fig. 9).^{20,21}

B. The Two-Stage Strategy: Chemoprevention and Chemotherapy

Due to the dual role of ROS in cancer development, both pro-oxidant- and antioxidant-based agents have been developed for cancer prevention and therapy.^{84,158,164–166} Pro-oxidant-based anticancer agents can not only directly increase ROS production but also decrease the antioxidant defense system of cancer cells. The antioxidant-based agents can directly scavenge intracellular ROS, enhancing ROS scavenging enzyme activities.

Chemoprevention and chemotherapy are different concepts and are useful based on which stage of cancer the cells are. Cancer chemoprevention could be achieved using antioxidant-based agents to prevent or reduce excessive cellular levels of oxidative stress, which inhibit malignant transformation of the normal cell conferred in the first stage. However, cancer chemotherapy may be achieved using prooxidant-based agents to increase the generation of ROS, which induce oxidative stress to kill cancer cells conferred in the second stage.

According to one report, 90–95% of cancers are caused by life style factors and only 5–10% are caused by genetic defects.¹⁶⁷ These proportions indicate that cancer is a disease that can be prevented largely by life style changes. Because ROS are involved in the transformation of nonmalignant cells to malignant cells, a potential approach to cancer prevention might be to control ROS production at the pretransformation stage. However, antioxidant-based cancer therapy has two caveats. First, the use of antioxidants can disturb the ROS dependent normal cell function and promote tumor growth, especially when ROS are required for apoptotic cell death of precancerous and transformed cells. Second, the use of antioxidants would interfere with radiotherapy and chemotherapy, which are largely dependent on ROS that induce cytotoxicity in tumors.^{168,169}

An opposite approach is to treat cancer cells with pharmacological agents that have pro-oxidant properties, which increases ROS generation or abrogates the cellular antioxidant systems. In preclinical models, ROS-generating agents showed selective toxicity in tumor cells with increased endogenous ROS, raising oxidative stress over the threshold of toxicity as antioxidant systems become overwhelmed.^{170–172} As ROS level appears heterogeneous even within one given type of cancer cells, a so-called “two-stage strategy” uses the opposite ROS-mediated approach sequentially to kill cancer cells more effectively. Our studies have shown that metal-induced ROS generation is oncogenic by inducing cell transformation in the early stage of metal carcinogenesis, nonetheless, decreased ROS generation is oncogenic by resulting in apoptosis resistance and increased cell survival in the late stage.^{14–16,19}

Therefore, the first stage mission is to activate antioxidant and decrease ROS in nontransformed cells to prevent metal-induced malignant transformation, while the second-stage mission is to activate oxidant and increase ROS in transformed cells to decrease

survival of these metal-transformed cells. Notably, the correct two-stage strategy contributes to a potent advantageous outcome and vice versa. Although chemoprevention and chemotherapy are based on different contexts, they have a common goal that focuses on suppressing metal carcinogenesis.

C. A Combination of ROS-Mediated Approaches is Very Successful

A combination approach based on the modulation of ROS stress and the breaking of signaling molecules associated with redox adaptation might be required to effectively eliminate cancer cells. In clinical treatment, there are some examples that are worth learning from: a combination of the ROS-generating agent arsenic trioxide and the SOD inhibitor 2-Me showed potent activity against primary chronic lymphocytic leukaemia (CLL) cells and significantly enhanced the cytotoxic activity of 2-Me in CLL cells that were resistant to 2-Me alone.¹⁷³ Combinations of arsenic trioxide and ascorbic acid-mediated GSH depletion were also shown to be clinically effective in the treatment of relapsed or refractory multiple myeloma.¹⁷⁴ Furthermore, recent work suggests that the transcription factor Nrf2 is responsible for resistance to the GSH-depleting agent BSO and abrogation of Nrf2 in combination with BSO seemed to be an effective therapeutic strategy.¹⁷⁵ Interestingly, the combination of ABT-737 (an inhibitor of the prosurvival molecule Bcl2) and 4HPR (which causes ROS generation) enhanced the mitochondrial apoptotic cascade with minimal cytotoxicity to normal lymphocytes.¹⁷⁶

As far as metal carcinogenesis is concerned, the combination of ROS-mediated therapy is consist of three steps: (1) in the early stage, antioxidants inhibit ROS in nontransformed cells to prevent metal-induced malignant transformation; (2) in the late stage, targeting the key molecule Nrf2 to break the redox adaptation and (3) using pro-oxidants to elevate the generation of ROS to kill cancer cells directly. This therapeutic strategy brings us some inspiration: combinations of ROS-generating agents with agents that can break the redox adaptation could be a better strategy for enhancing cancer cell cytotoxicity. The two-stage strategy focuses on chemoprevention and chemotherapy with different context in different stages. However, for integral pathology process of metal carcinogenesis, we should develop a drug-combination plan to effectively eliminate cancer cells. The combination of ROS-mediated approaches would be derived from the two-stage strategy. According to this presence of dual roles of ROS in metal carcinogenesis, it appears that the prooxidant and antioxidant functions could be used to cooperatively treat a common cancer in two stages.

D. Combination of ROS-Mediated Approaches vs. Drug Resistance

One of the major hurdles in treating cancer is that tumor cells, although initially sensitive, gradually develop resistance to chemotherapy, in part due to the induction of multidrug-resistance proteins. However, there is an obvious difference between common cancer and metal-induced cancer in the underlying mechanism of drug resistance. The drug resistance of metal carcinogenesis is attributed to redox adaption resulting from constitutively high overexpression of Nrf2. Extensive research over the past several years has indicated that ROS-generating anticancer agents can reduce the drug resistance of cancer cells because excessive ROS can lead to cell death through several mechanisms, including apoptosis, necrosis, and autophagy.^{177–180}

In this regard, we should break the redox adaptation by targeting Nrf2 first, and we should take advantage of a pro-oxidative strategy that will not only kill cancer cell directly but also sensitize tumor cells to chemotherapeutic agents. This “one stone, two birds” strategy shows promising prospects in a wide variety of cancer therapies.¹⁷⁸ Therefore, we recommend a good pro-oxidative agent: arsenic trioxide (As_2O_3). It is well known that arsenic is a toxic compound, and it has been listed a human carcinogen for many years.¹⁸¹ Although arsenic can be poisonous and chronic arsenic exposure from industrial or natural sources can cause lung cancer, arsenic has been used therapeutically for more than 2,400 years.^{182,183} Arsenic trioxide can induce apoptotic or autophagic cell death, or both, in various types of cancer cells, and although not all studies have addressed the role of oxidative stress, several have demonstrated that ROS formation is essential for As_2O_3 cytotoxicity.^{184,185} Arsenic trioxide is another approved anticancer agent that has shown potential against acute promyelocytic leukemia. As_2O_3 has the ability to induce superoxide production in cancer cells.^{186–189}

Why do we discuss arsenic trioxide in particular? Because arsenic trioxide is a solid example for presenting on the dual role of ROS in metal carcinogenesis. Thus, we should take advantage of the pro-oxidative property of arsenic trioxide, which could result in two anticipated results: (1) owing to an anticancer effect, the arsenic-induced carcinogenesis could be self-limited, but the precondition is to abrogate redox adaptation by downregulating Nrf2; (2) arsenic trioxide kills cancer cells and alters drug resistance with appropriate dosing. Collectively, it is intriguing that the metal induced ROS, due to its oncogenic role, could become a key helper through its antioncogenic properties (Fig. 10).

E. Distinguishing the Different Stages is the Most Essential Step in Metal Carcinogenesis

We have demonstrated that cancer chemoprevention and chemotherapy are different concepts and must be used accordingly in the different contexts of the different stages. Therefore, distinguishing the different stages is the most essential step in metal carcinogenesis. We have indicated that the dual roles of ROS are dependent on the different amounts of ROS in different stages. Measuring the magnitude of ROS would be a promising method to define the different phases. In addition, the assessment of redox state of body fluids may be helpful in predicting efficacy and side effects. Although it is simple to detect the hydrogen peroxide level in body fluids such as human urine, the available data are currently insufficient to support the use of urinary H_2O_2 as a biomarker of oxidative stress.⁷² However, at the cellular level, small-molecule fluorescent probes are vital tools for monitoring reactive oxygen species.¹⁹⁰ The adverse properties of ROS with respect to their detection, such as their short lifetimes and the competitive presence of various endogenous reactive species, capable of interfering with the probe in biological matrices, have hindered the effective performance of most probes and have complicated the design of suitable probes.^{191–193} Although there are disadvantages in specificity, semiconductor quantum dots (QDs) fluorescent probes have been capable of circumventing some problems and attracted significant attention among the various probes developed to date.¹⁹³

We can measure the ROS magnitude through QD fluorescent probes. A high ROS measurement would tell us that the cell is in the early stage; a low ROS measurement would tell us that the cell is in the late stage. However, a property of luteolin exhibits both

antioxidant and pro-oxidant activities, allowing it to act in an antioncogenic role in the same cancer pathology process. Thus, we may not need to know which stage the cancer cells are before administrating luteolin as an anticancer therapy. This finding property provides avenues of exploration for other natural compounds similar to luteolin that can enrich our anticancer tools. Above all, distinguishing the different stages by measurement of ROS is a helpful approach in fighting against cancer effectively—not only prevention but also therapy.

F. A Novel Strategy for Breaking the Redox Adaptation in Metal-Induced Cancer Stem Cells

The number of studies on cancer stem cells has steadily increased. A small subpopulation of primary cancer cells expressing stem cell properties are resistant to certain chemotherapeutic agents and radiation.^{194,195} Considering the biological properties of cancer stem cells, this unique cell subpopulation might have a high antioxidant capacity to keep cellular ROS at lower levels and maintain both stemness and cancer-forming capabilities.^{196,197} The highly upregulated antioxidant mechanisms might contribute to cancer stem cell survival and drug resistance. Additionally, some stem cells reside in a relatively low oxygen microenvironment, which could also limit the endogenous ROS production.¹⁹⁸ Thus, cancer stem cells may be relatively resistant to radiotherapy and chemotherapy with ROS-mediated agents, the cytotoxicity of which requires the availability of local oxygen.

With regard to metal carcinogenesis, cancer stem cells are relatively new topic. Our unpublished data show that in nickel-induced cancer stem-like cells, superoxide dismutase and catalase are upregulated, leading to a reduced ROS level and contributing to stemness properties. This study also showed that overexpression of SOD1 could increase stemness properties. Pharmacological depletion of SOD1 functions markedly decreases the accumulation of cancer stem-like cells in nickel induced transformed cells.¹⁷

We have acquired an understanding of the dual role of oxidative stress in metal carcinogenesis. Breaking redox adaption appears to be the most critical step for significant therapeutic implications in metal carcinogenesis. Moreover, we have displayed a series of strategies to quench the “redox adaption” by generating ROS: targeting Nrf2, the two-stage strategy, and drug combination therapy. These approaches might provide a possibility to abrogate the survival mechanisms in cancer stem cells and enable the elimination of the root of cancer. The main approaches to eliminate cancer stem cells lie in four points in metal carcinogenesis: (1) inhibition of the antioxidant systems in cancer stem cells sensitizes them to other anticancer agents; (2) enhancement of exogenous ROS stress to cause cancer stem cells to lose their stemness; (3) combination of a redox-modulating strategy with standard anticancer agents to be more effective in killing cancer stem cells; and (4) reversal of the low oxygen microenvironment against cancer stem cells (Fig. 8).

VIII. ANOTHER PROMISING NATURAL DIETARY COMPOUND: PIPERLONGUMINE

Piperlongumine is a natural compound from the fruit of the long pepper, a pepper plant found in southern India and southeast Asia.¹⁹⁹ Recently, piperlongumine’s anticancer

properties have been widely reported.^{200–202} In a recent report, various human cells transformed to express cancer phenotypes were able to generate ROS in response to the small-molecule piperlongumine, whereas, normal human cells could not generate ROS with the same treatment.¹⁹⁹

In our ongoing study, we have found that in human lung bronchial epithelial cells, piperlongumine activates inducible Nrf2, leading to an increase in antioxidants, a decrease in ROS, and inhibition of Cr(VI)-induced malignant cell transformation. In Cr(VI)-transformed cells, constitutive EGFR activation initiates autophagy and causes autophagy deficiency by blocking the fusion between autophagosomes and lysosomes. This blockage results in constitutive activations of p62 and Nrf2, leading to development of apoptosis resistance and tumorigenesis.¹⁹ Piperlongumine is able to counteract this tumorigenic process by suppressing constitutive EGFR activation, deficiency of autophagy constitutive Nrf2 activation and increasing ROS.

This finding of new nutraceutical confers us three compelling benefits: (1) it illustrates that constitutive EGFR activation is a upstream molecular pathway of the formation of autophagy deficiency in metal carcinogenesis; (2) it illustrates that targeting constitutive EGFR reverses constitutive activations of p62 and Nrf2, leading to downregulation of apoptosis resistance and tumorigenesis; and (3) piperlongumine is an antioncogenic agent similar to luteolin, which can exhibit both antioxidant and pro-oxidant roles in the cancer therapeutic process.

IX. SUMMARY AND FUTURE PERSPECTIVES

Although the molecular mechanisms are not completely understood, the potential of metals to generate reactive oxygen species (ROS) and thus to alter cellular redox status is considered the most important mechanism involved in metal-induced carcinogenesis. ROS are ubiquitous in all mammalian cells, and some ROS are required by both cancer cells and normal cells for normal signaling. However, some types of ROS are toxic to both cancer cells and normal cells under certain physiological conditions. Importantly, ROS plays a dual role in metal carcinogenesis; they not only can play an oncogenic role but also can act in an antioncogenic role.

Consequently, the ‘double-edged sword’ of ROS and how to effectively and selectively kill tumor cells has emerged as one of the major concerns for further employment of ROS-manipulated approaches in metal carcinogenesis. This review is based on the dual role of ROS and the mechanism of formation of metal carcinogenesis in different stages including normal cell transformation, autophagy deficiency, and the p62/Nrf2 positive feedback loop with apoptosis resistance. Focusing on Nrf2, we have exhibited it as the most important key molecule for the dual role of ROS in metal carcinogenesis, and we have also presented a potentially safe and effective weapon, luteolin, aimed to enhance dual roles of Nrf2 in chemoprevention and chemotherapy.

In our review, we have also discussed the relationship between redox adaption and the dual role of ROS in metal carcinogenesis. Moreover, we have demonstrated therapeutic strategies

to break the “redox adaption” including the “two stages strategy:” drug-resistance abrogation and drug combination therapy. These new strategies are innovative not only in the area of metal carcinogenesis but also in the cancer field as a whole. These new ideas can help broaden our knowledge in the field of ROS cancer biology, including ROS-based cancer prevention, and move this field forward scientifically. Furthermore, these new ideas involve transdisciplinary collaboration in the areas of autophagy, apoptosis, cancer chemoprevention/chemotherapy, and cancer drug resistance, which will be helpful in the formulation of an expert consensus or guidelines on treatment strategies of metal carcinogenesis.

Certainly, by exploiting the dual role of ROS, we have found new areas to explore in future research projects. First, because arsenic trioxide has a pro-oxidative property that can be utilized to kill cancer cells, we should also take advantage of its antioncogenic ability to inhibit metal-induced cancer in the late stage. Second, measuring the amount of ROS in different contexts appears to be critical for proper cancer chemoprevention and chemotherapy, and this area still needs further insight and research. Third, considering the crucial role of cancer stem cells in tumor initiation, recurrence and drug resistance, the potential of using a redox-modulating strategy to eliminate this subpopulation of malignant cells has significant promise in metal-induced cancer treatments. Above all, these ideas are going to become our new inspiration and challenge while providing new opportunities and hope in the next era of metal carcinogenesis.

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FIG. 1:

Mechanisms of metal carcinogenesis. Three major mechanisms apply for the metal carcinogenesis: oxidative stress, DNA repair modulation and disturbances of redox-sensitive signal transduction pathways. The induction of oxidative stress is the most attractive hypothesis to explain mutagenic and carcinogenic effects of metals. Intracellular superoxide ($O_2^{\bullet-}$) is primarily produced by the oxidation of NADPH and mitochondria. Superoxide is rapidly converted into hydrogen peroxide (H_2O_2) by SOD. Highly reactive $\bullet OH$ is generated via Fenton and/or Haber–Weiss reactions by the breakdown of H_2O_2 . Some metal ions are also effective catalysts for Fenton and Fenton-type reactions. Oxidative DNA damage induced by $\bullet OH$ is a key factor influencing the mutagenic and carcinogenic load in living organisms with metals. H_2O_2 may be converted to H_2O by cellular antioxidant proteins, such as glutathione peroxidase (GPx), and catalase (CAT). Metals can inhibit these antioxidants. The repair of DNA damage can be inhibited by a diversity of metals. Alternatively, the mechanisms of metal carcinogenesis may involve multiple pathways to affect redox-sensitive signal transduction, which contributes to metal carcinogenesis.

FIG. 2:

ROS domains vary based on cells. ROS has three domains depending on the cell types. The first domain is the “physiological and pathological function of ROS” which applies to all cells, the second domain is the most important, “the ‘double-edged sword’ property of ROS” which applies to cancer cells, and the last domain is the “dual roles of ROS and the redox adaptation” which is most important to metal-induced cancer cells.

FIG. 3: ROS levels in different stages. In metal carcinogenesis, amount of ROS is in a state of constant change. The dual roles of ROS are dependent on the different amount of ROS in different stages. The oncogenic role is related to higher ROS level in early stage, and antioncogenic role is related to lower ROS level in late stage respectively. Furthermore, if the increase of ROS reaches a certain threshold level that is incompatible with cellular survival, ROS may exert a cytotoxic effect, leading to the death of malignant cells and thus limiting cancer progression. Different amount of ROS leads to different destinations in metal carcinogenesis.

The difference of two stages

	Background	Cell	Nrf2	Anti-oxidant	Drug-resistance	ROS Level	Bcl-2 Increase	Apoptosis resistance	ROS role
Early stage	Oxidative Stress	Normal to Transformed	Inducible	Decrease	Negative	Excessive	Negative	Negative	Oncogenic
Late stage	Autophagy deficiency	Transformed to Tumor	Constitutive	Increase	Positive	Fewer	Positive	Positive	Anti-oncogenic

FIG. 4:

The key differences in two stages. There are two stages in metal carcinogenesis. This figure compares the key differences in two stages. The late stage is more unique than early stage, because the mechanism of Nrf2 signaling not only activates ROS-scavenging systems but also inhibit apoptosis by upgraded Bcl-2.

FIG. 5:

The dual role of Nrf2. Activation of inducible Nrf2 is anticarcinogenic in the early stage of carcinogenesis (from normal cells to malignantly transformed cells) by upregulation of its target antioxidants to reduce the levels of ROS, which is desirable. Otherwise, constitutive expression of Nrf2 is tumorigenic role through a protection to cancer cells from oxidative stress and contributes to up-regulation of Bcl-2 and Bcl-XL and development of resistance to apoptosis in the late stages of carcinogenesis.

FIG. 6:

The pathway of autophagy deficiency and constitutive Nrf2 activation. The pathway of autophagy deficiency and constitutive Nrf2 activation is very important to formation of dual role of oxidative stress in metal carcinogenesis. For autophagy deficiency, this process consists of two steps: the first step is initiation of autophagy by decrease in mammalian target of rapamycin (mTOR), autophagy inhibitor, and increases in transcription factor EB (TFEB), Beclin-1, and autophagy-related protein 7 (ATG7), the second step is the blockage of the fusion between autophagosomes and lysosomes by inhibition of LAMP2a. Both of two steps are indispensable and originated from constitutive EGFR activation. In turn, autophagy deficiency would trigger accumulation of p62, which activates a positive feedback loop among p62, Nrf2 and keap1 followed by constitutive Nrf2 signal activation and carcinogenesis.

FIG. 7: Luteolin is a ‘double-edged sword.’ Luteolin has two types of properties: (a) to activate inducible Nrf2 and decrease ROS in normal cells to prevent metal-induced malignant transformation and (b) to inhibit constitutive Nrf2 activation and increase ROS and decrease Bcl-2 in metal-transformed cells to suppress survival of these transformed cells and to prevent from tumorigenesis.

FIG. 8: Mind mapping of acquiring enlightenments from dual role of ROS in metal carcinogenesis. By exploiting the two roles of ROS, we have acquired possible therapeutic developments for metal carcinogenesis. This mapping exhibits a logical relationship and speculates a series of strategies to break the “redox adaption” including of two-stage strategy and drug combination. The combination of ROS-mediated approaches derived from the two-stage strategy is a crucial step, which would fight against drug resistance and cancer cells.

FIG. 9: Redox adaptation and drug resistance. Some exogenous ROS act as an insult (pro-oxidative agent) on normal cell, regular cancer cell, and metal-induced cancer cell, which can result in different consequences. First, under physiological conditions, normal cells maintain redox homeostasis with a low level of basal ROS by controlling the balance between ROS generation (pro-oxidants) and elimination (antioxidant capacity), which can be mobilized to prevent the ROS level from reaching the cell death threshold (labeled dashed line in figure). Second, a further increase of ROS stress in cancer cells using exogenous ROS-modulating agents is likely to cause elevation of ROS above the cell death threshold level. Regular cancer cells are more vulnerable to further oxidative insults induced by ROS-generating agents. Third, redox adaptation through the elevation of endogenous antioxidants and the activation of cell survival pathways may confer an increased capacity to tolerate exogenous stress and insults, a decrease in apoptotic execution, is a common phenomenon in metal-induced cancer cells. The increased capacity of redox adaptation in cancer cells has been linked to drug resistance. Some exogenous ROS insult (pro-oxidative agent) act on metal-induced cancer cell, which trigger an increasing of ROS but cannot reach the cell death threshold.

FIG. 10:

A combination of ROS-mediated approaches. Cellular redox homeostasis is maintained by the balance between ROS generation and elimination. Exogenous agents that increase ROS generation or decrease antioxidant capacity that increase ROS generation will shift the redox balance and result in an overall increase in the level of ROS, which when above a cellular tolerability threshold may induce cancer cell death. A combination of ROS-mediated approaches has been found to be very effective and selective in late stage. Combination of luteolin and pro-oxidative agent, first step: luteolin targets Nrf2 to restore the normal level of ROS by abrogating the redox adaptation. Next step: pro-oxidative agents kill cancer cells by elevating the generation of ROS.