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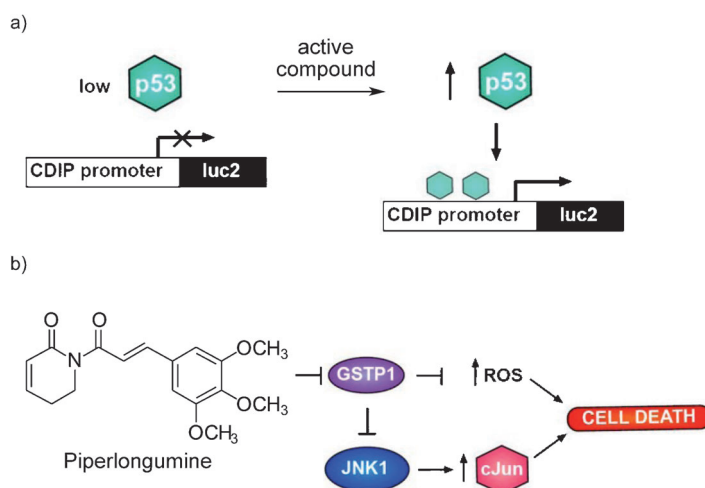
Runaway ROS as a Selective Anticancer Strategy

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The majority of cancer therapies target macromolecules important for general cell survival, such as DNA, topoisomerase, and tubulin (e.g., cisplatin, doxorubicin, and paclitaxel, respectively). While these agents are effective at killing many types of cancer cells, they also affect rapidly dividing normal cells, such as the intestinal lining and bone marrow, resulting in dose limiting toxicities that reduce efficacy against many solid tumors and metastatic disease. A goal of personalized therapy as applied to cancer is to understand the precise defects in the cancer cell, which may only be present in one particular type of cancer and/or a small subset of patients, and to have drugs that exploit these differences. A small number of such cancer-specific therapies exist. These treatments commonly target a unique characteristic of cancer, such as a translocation, mutation, or a protein with elevated expression.^[1] The poster child for this approach is imatinib (Gleevec), which inhibits the tyrosine kinase domain of the Bcr-abl protein, the product of a translocation between chromosomes 9 and 22 often observed in patients with chronic myeloid leukemia. Analogous success stories have appeared, including gefitinib (Iressa), which inhibits a mutated version of the epidermal growth factor receptor (EGFR) protein found in some non-small-cell lung cancers,^[1,2] and trastuzumab (Herceptin), a monoclonal antibody that targets the Her-2/neu receptor that is overexpressed in some breast cancers.^[3] As impressive as these success stories are, identifying enough of these types of compounds to cover the diversity of differentially expressed cancer targets is a daunting task. Ideally, compounds could be identified that are capable of killing cancer regardless of tissue origin or cancer subtypes, as is seen with cytotoxins, but with the cancer specificity observed with more personalized therapies, such as those described above.

In a recent issue of *Nature*, Raj et al. disclose a compound that kills cancer cells of various origins in a highly specific manner over noncancerous cells.^[4] They identified this compound using a phenotypic screen, demonstrated its selectivity for cancer cells and efficacy in murine tumor models, and discovered the targets of the compound using a combination of stable isotope labeling with amino acids in cell culture (SILAC) and quantitative proteomics. The phenotypic screen identified

compounds that increase expression of a luciferase reporter gene that is located behind a p53-activated promoter for a known proapoptotic gene (Scheme 1 a). From this screen, pi-



Scheme 1. In a cell-based screen, U2OS cells were stably transfected with a construct containing the p53 binding portion of the promoter for the pro-apoptotic protein CDIP upstream of the *luc2* reporter gene. These cells have low p53 levels ultimately resulting in low expression of the luciferase protein. Active compounds will upregulate p53, which subsequently binds the CDIP promoter, activating transcription of *luc2* and ultimately translation of the luciferase protein. b) Proposed mode of action: piperlongumine inhibits glutathione S-transferase pi 1 (GSTP1), a protein known to reduce oxidative stress within cells. Inhibition of GSTP1 leads to greater levels of reactive oxygen species (ROS) in cells and ultimately cell death. Additionally, GSTP1 inhibits activation of JNK1, preventing apoptosis.^[19,20] By inhibiting GSTP1, piperlongumine might cause the activation of JNK1 and ultimately apoptosis.

perlongumine (Scheme 1 b) was found to induce a high luciferase signal, suggesting that it activates p53-dependent apoptosis. Further studies revealed that piperlongumine also kills cancer cells with mutant p53.

While the ability of piperlongumine to kill cells regardless of p53 status makes it an attractive anticancer compound, the phenotype that made this compound especially worthy of further study was the observation that piperlongumine selectively induces death in cancer cell lines compared with normal cells—Raj et al. reported that piperlongumine reduced the viability of 14 cancer cell lines of different origins, with an average IC₅₀ value of ~7 μM, but did not affect six different noncancerous cell types at concentrations as high as 15 μM (highest concentration tested). Additionally, noncancerous cell lines became sensitized to piperlongumine after oncogenic conversion using different oncogenes, further implying that the mode of action of piperlongumine is selective for cancer but not limited to a specific cancer subtype. Similar effects were

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observed *in vivo*, with piperlongumine impressively reducing tumor burden in murine models of melanoma, as well as bladder, breast, and lung cancers, while having little to no effect on normal mouse tissue.^[4]

From these experiments, piperlongumine was found to be a selective, if not a terribly potent (in cell culture), inducer of cancer cell death. The authors then set off to determine how piperlongumine selectively induces death in cancer cells. Piperlongumine is an amide alkaloid found in *Piper tuberculatum*, a pepper plant found in Northeast Brazil.^[5] Traditionally, this plant has been used in folk medicine as an analgesic, sedative, and an antidote for snake bites.^[5] More recently, piperlongumine has been found to decrease platelet aggregation, as well as act as an analgesic and anti-inflammatory agent likely through an interaction with the cyclooxygenase pathway.^[5,6] Additionally, piperlongumine has previously been reported to be cytotoxic to tumor cells.^[7,8] These studies demonstrated that piperlongumine can induce either apoptosis or necrosis, but no conclusive mode of action was identified.^[7] Raj et al. were able to identify putative targets of piperlongumine through a combination of SILAC and quantitative proteomics. In SILAC, cells are cultured in media containing either ¹²C- or ¹³C-labeled amino acids, resulting in the production of "light" or "heavy" proteins.^[9] For the identification of compound targets, the proteins are individually incubated with beads containing the compound of interest.^[10] The heavy proteins are mixed with beads containing compound, while the light proteins are mixed with beads containing compound along with free compound to competitively bind the protein of interest. After extensive washes, the proteins are eluted off the beads, mixed and digested, then identified and quantified by mass spectrometry (MS). Any proteins that nonspecifically bind the beads should show up in approximately equal amounts, while those that specifically bind the compound of interest should have greater amounts of heavy protein.

Using this method, Raj et al. identified 12 potential targets for piperlongumine that were conserved across two cell lines. Seven of the potential targets are involved in cellular response to oxidative stress. The two highest hits were glutathione S-transferase pi 1 (GSTP1) and carbonyl reductase (CBR1), both of which are known to detoxify xenobiotics.^[11,12] Additionally, GSTP1 is involved in the activation of other antioxidant proteins, such as peroxiredoxin 1 (PRDX1).^[11] Piperlongumine was shown to modestly inhibit the enzymatic activity of recombinant GSTP1 *in vitro*, consistent with the idea that GSTP1 is a target of piperlongumine. Additionally, treatment with piperlongumine decreased glutathione (GSH) levels while increasing reactive oxygen species (ROS) in multiple human cell lines, further supporting a ROS-dependent mechanism (Scheme 1 b).

The ROS stress response exists in all cells, so how does this compound kill cancer cells selectively? It is well known that cancer cells have higher oxidative stress levels, likely due to increased metabolism, mutations in the electron transport chain (ETC), and activation of certain oncogenes.^[13,14] To a certain extent, ROS actually aids in tumorigenesis, promoting further mutations, cell proliferation, angiogenesis, and metastasis, as well as preventing apoptosis.^[15,16] However, because cancer cells have higher levels of ROS, they are also more sensitive to increases in oxidative stress.^[14] Multiple drugs and drug candidates seek to take advantage of this either by direct generation of ROS or inhibition of stress response (Figure 1). ROS-generating compounds typically produce ROS either through disruption of ETC or bioactivation of compounds (e.g., quinones) leading to unstable intermediates (e.g., semiquinones or hydroquinones) that produce ROS by redox cycling (Figure 1 a).^[14,17] Inhibition of the stress response pathways can be achieved through depletion of glutathione (GSH) or inhibition of proteins directly involved in ROS detoxification, such as superoxide dismutases (SOD) (Figure 1 b). Given the cumulative evidence, Raj et al. conclude that piperlongumine induces ROS

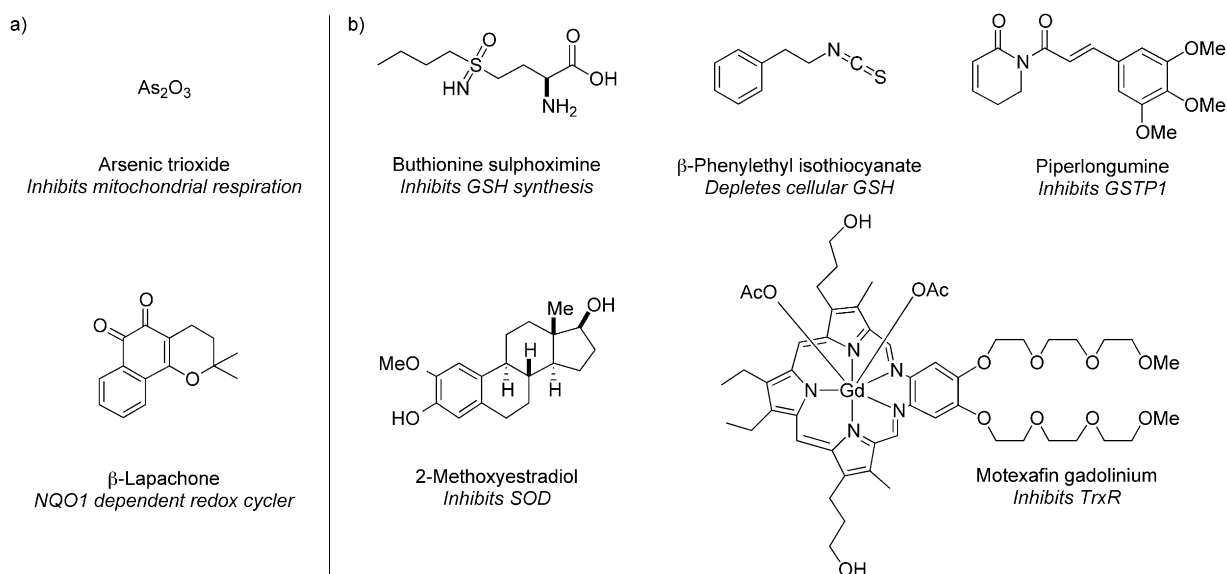


Figure 1. ROS-dependent drugs and drug candidates. a) ROS generators are compounds that directly increase cellular ROS levels. b) Inhibitors of stress responses are compounds that either deplete substrates such as glutathione (GSH) or inhibit enzymes such as superoxide dismutase (SOD) or thioredoxine reductase (TrxR), which are necessary for controlling ROS levels. Below each compound is the name and the proposed mechanism of action.^[14]

selectively in cancer cells, presumably by its effects on stress response enzymes such as GSTP1 and CRB1.

Interestingly, the authors suggest that piperlongumine induces apoptotic cell death; this would be in contrast to the type of cell death induced by some other ROS-generating compounds. For example, the experimental therapeutic β -lapachone is bioreductively activated by the enzyme NQO1 and generates ROS through subsequent redox cycling.^[17] Strong evidence points to β -lapachone killing cells not through the classical apoptotic pathway, but through PARP-1-dependent cell death, sometimes called parthanatos.^[18] The implication that differences in ROS generation may activate disparate cell death mechanisms is an intriguing observation that is certainly an avenue for future research.

The mode of action studies with piperlongumine shine a light on the potential of GSTP1 inhibition as an anticancer strategy, specifically through ROS generation. However, GSTP1 has another function: the inactivation of the pro-apoptotic protein c-Jun N-terminal Kinase (JNK1).^[19] Inhibition of this interaction results in activation of c-Jun, the activator protein-1 (AP-1) transcription factor, and ultimately the transcription of genes involved in cell death. For this reason, inhibition of GSTP1 is an emerging target for novel chemotherapeutics, and several very potent inhibitors of GSTP1 have been described.^[20] One of these, the peptide TLK199 (ezatiostat hydrochloride) was shown to potentiate toxicity of chlorambucil in cell culture.^[20] TLK199 has been evaluated in human clinical trials as a treatment for myelodysplastic syndrome (MDS) where it has shown efficacy.^[20,21] It would be interesting to evaluate piperlongumine side by side with some of these well-characterized GSTP1 inhibitors in cell culture experiments. The fact that piperlongumine is only modestly potent in vitro as a GSTP1 enzyme inhibitor (~50% reduction of activity with 100 μ M compound) might suggest that some of the other targets that Raj and co-workers identify through SILAC could also be relevant to the mode of action of piperlongumine, or that piperlongumine is converted in the cell to a more potent GSTP1 inhibitor.

The discovery of piperlongumine by Raj et al. demonstrates how small-molecule screening followed by sophisticated target identification methods can be used to identify leads for novel and selective treatments for cancer. While the modest solubility of piperlongumine will need to be improved as it moves towards a clinical trial, its activity against a wide variety of cancer cell lines, its impressive efficacy in multiple murine tumor

models, and its selectivity for cancerous over noncancerous tissues make it a promising anticancer candidate.

Keywords: cancer · drug discovery · reactive oxygen species (ROS) · small-molecule screening · target identification

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