

Addendum

PERK-dependent regulation of HSP70 expression and the regulation of autophagy

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Abbreviations: ERK, extracellular regulated kinase; MEK, mitogen activated extracellular regulated kinase; EGF, epidermal growth factor; OSU, OSU-03012; PARP, poly ADP ribosyl polymerase; PI3K, phosphatidylinositol 3 kinase; -/-, null / gene deleted; ERK, extracellular regulated kinase; MAPK, mitogen activated protein kinase; MEK, mitogen activated extracellular regulated kinase; R, receptor; JNK, c-Jun NH₂-terminal kinase; dn, dominant negative; COX, cyclooxygenase; P, phospho-; ca, constitutively active; WT, wild type; PERK, PKR like endoplasmic reticulum kinase; HSP, heat shock protein

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The manuscript by Park et al. (Mol Pharm 2008; mol.107.042697/PMID: 18182481) further defines the mechanism(s) by which OSU-03012 (OSU) kills transformed cells. It notes that in PKR-like endoplasmic reticulum kinase null cells (PERK^{-/-}) the lethality of OSU is attenuated. OSU enhances the expression of ATG5 in a PERK-dependent fashion and promotes the ATG5-dependent formation of vesicles containing LC3, followed by a subsequent cleavage of cathepsin B and a cathepsin B-dependent formation of low pH intracellular vesicles; cathepsin B is activated and released into the cytosol, and genetic suppression of cathepsin B or AIF function significantly suppresses cell killing. In parallel, OSU causes PERK-dependent increases in HSP70 expression and decreases in HSP90 and Grp78/BiP expression. Inhibition of HSP70 expression enhances OSU toxicity and overexpression of HSP70 suppresses OSU-induced low pH vesicle formation and lethality. Thus, in this system PERK signaling promotes autophagy, which is causally linked to lysosomal dysfunction, cathepsin activation and cell death. However, in parallel, PERK signaling acts to suppress autophagy and lysosomal dysfunction by increasing the expression of HSP70. These findings may help explain why, in

a cell type and stimulus-dependent fashion; autophagy has been noted to act either as a protective or as a toxic signal in cells.

Inhibitors of cyclooxygenase 2 (COX2) were originally developed to inhibit inflammatory immune responses, with a primary intention to use such agents clinically in the treatment of chronic diseases, e.g., rheumatoid arthritis.¹ It was also noted that COX2 is overexpressed in many tumor cells and that agents which inhibit COX2, e.g., CelecoxibTM (CelebrexTM) can suppress tumor cell growth in vitro and when grown as xenografts in animals.^{2,3} The agent OSU-03012 was developed as an anti-cancer agent, with Celecoxib as the chemical backbone.⁴ In vitro OSU-03012 has an order of magnitude greater tumoricidal activity than Celecoxib, but lacks COX2 inhibitory activity. Our recent data argue that OSU-03012 causes cell death through mechanisms which involves a form of endoplasmic reticulum (ER) stress signaling and mitochondrial dysfunction but that are a caspase-independent form of cell death, as initially judged by a lack of effect of the caspase inhibitor zVAD and expression of dominant negative caspase 9. Instead, our findings argue in HCT116 cells that knock down of apoptosis inducing factor (AIF) expression significantly attenuates OSU-03012 lethality.⁵

The toxicity of OSU-03012 in tumor cells was initially argued to be due to inhibition of the enzyme PDK-1, a kinase intermediary within the PI3 kinase pathway, in as much as OSU-03012 can suppress AKT (S308) phosphorylation and shows measurable inhibition of PDK-1 activity in the 5–50 μM range in vitro.⁴ OSU-03012 has also been shown to interact in a synergistic fashion with BCR-ABL inhibitors and with the ERBB2 inhibitor Herceptin to suppress tumor cell viability and to kill in a manner that is in many cell types at least partially caspase-independent.⁶⁻⁹ In our previous studies, we also noted that inhibition of either MEK1/2 or PI3K enhances the toxicity of OSU-03012 in glioma, colon cancer and transformed rodent fibroblast cell types.⁵ However, while

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OSU-03012 can suppress PDK-1 function and AKT activity, other data have also strongly argued that OSU-03012 toxicity, and its radiosensitizing effects, could not be attributed to suppression of AKT signaling in comparison to siRNA-mediated knock down of PDK-1 protein levels or dominant negative AKT expression.^{5,10} In the studies by Park et al, we examine in greater detail the impact of OSU-03012 on cell survival and define the molecular mechanisms by which OSU-03012 enhances tumor cell death.¹¹

In Park et al, we demonstrate that OSU-03012 promotes a dose-dependent induction of transformed cell killing that is significantly reduced in cells in which BID or AIF expression is transiently or stably suppressed. Of note, however, is that at in vitro drug doses > 3 μ M, even knock down of AIF expression only partially maintained cell survival in our analyses, arguing for multiple over-lapping modes of OSU-03012 drug-induced toxicity. Similarly, molecular or small molecule inhibition of cathepsin B function only strongly suppresses OSU-03012 toxicity at lower drug doses. Recently, studies in human myeloma cells using approximately one order of magnitude higher concentrations of OSU-03012, report that cell killing is not inhibited by either caspase or cathepsin inhibitors.¹² These studies also note that BID cleavage occurs in response to OSU-03012 exposure. In Park et al, we find that the drug activated the ER resident caspase enzymes pro-caspase 2 and pro-caspase 4; as both caspase 2 and caspase 4 have been shown to cleave BID, independently of cathepsin protease action, it is possible that some cells may primarily utilize caspases 2 and 4, which are generally insensitive to "pan" caspase inhibitors such as zVAD, to process BID into its active form after OSU-03012 exposure rather than cathepsin proteases. Thus, it is an important point to note that our AIF, cathepsin B and autophagy findings likely represent a primary mode of OSU-03012 toxicity, but one which may become obscured at higher concentrations of this drug as multiple other mechanisms of drug-induced toxicity are induced. It is also pertinent to point out that at these lower drug concentrations, OSU-03012 marginally alters AKT (S308) phosphorylation suggesting PDK-1 inhibition is not a primary mode of drug toxicity.

We demonstrate that low dose OSU-03012 treatment causes a rapid PERK-dependent induction of intracellular vesicles in human cancer cells and in rodent fibroblasts that are associated with a transfected GFP-tagged LC3 protein. Knockdown of ATG5 expression significantly reduces the PERK-dependent induction of vesicles that are associated with the transfected GFP-tagged LC3 protein. OSU-03012 treatment also causes a rapid PERK-dependent and ATG5-dependent induction of tumor cell killing. This argues that OSU-03012 exposure causes an early PERK-dependent and ATG5-dependent autophagic response in transformed cell types that precedes the related AIF release into the cytosol, and morphological manifestation of cell death. Hence in our cell system, treated with low concentrations of OSU-03012, ER stress signaling from PERK, via ATG5 and autophagy, is causal in the pathway to cell death (Fig. 1).

In the analyses by Park et al we note that OSU-03012 treatment of transformed human and rodent cells initially increases expression of HSP70, in parallel to the formation of autophagosomes, in a PERK-dependent fashion, which based on established dogma within their respective fields are both putatively protective effects. However, OSU-03012 treatment of cells then subsequently causes a decrease in HSP90 and BiP/Grp78 expression in parallel to the release of

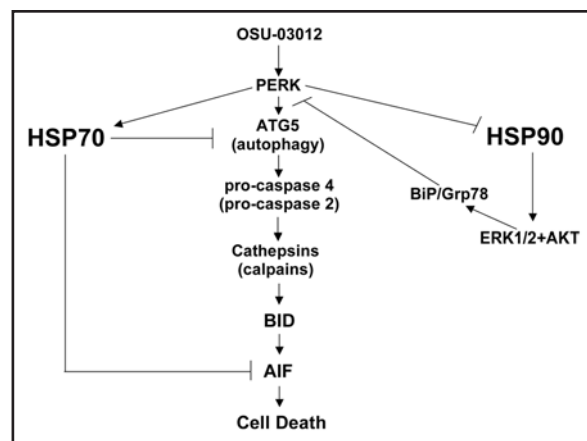


Figure 1. A molecular model by which OSU-03012 causes autophagy and by which PERK promotes and suppresses cell killing. OSU-03012 treatment activates PERK. PERK signaling increases HSP70 levels and depresses HSP90 levels. OSU-03012 also increases ATG5 levels and causes ATG5-dependent vacuolization and colocalization of a GFP-LC3 construct, indicative of autophagy. Autophagy leads to cathepsin-dependent cleavage of BID, and BID-dependent release of AIF into the cytosol. Over-expression of HSP70 suppresses autophagy as well as the toxicity of AIF. Depression of HSP90 levels will lead to a late phase reduction in the activity of ERK1/2 and AKT. Lower ERK1/2 levels will lead to lower BiP/Grp78 expression promoting hyper-PERK signaling, which may be highly toxic.¹⁹

cathepsin B and AIF into the cytosol, all of which are putatively toxic effects, and these effects also occur in a PERK-dependent fashion. Overexpression of HSP70 suppresses, and knockdown of HSP70 levels enhances, OSU-03012 toxicity. In our analyses, we also demonstrate that overexpression of HSP70 blocks the formation of GFP-LC3 vesicles and of low pH acidic endosomes following OSU-03012 exposure, demonstrating that one site at which HSP70 acts to promote cell survival following OSU-03012 treatment is at the earliest stages of ER stress signaling, at the level of autophagy induction. Collectively these findings support the hypothesis that OSU-03012 causes a form of ER stress which induces a protective PERK-dependent response involving elevated expression of HSP70 and autophagic vesicle formation. This putatively protective response ultimately degenerates, however, possibly due to prolonged PERK signaling and/or other factors such as the collapse of activity within multiple cyto-protective signaling pathways as cells begin to become dysfunctional, into a cytotoxic response with the formation of low pH acidic endosomes, reduced expression of HSP90 and BiP/Grp78, and the release of cathepsin B and AIF into the cytosol, which causes a nonapoptotic form of transformed cell death.

There is an additional possible discussion point for our findings with respect to HSP90, HSP70 and OSU-03012. Inhibition of HSP90 function has been shown by many laboratories to cause a reciprocal rise in the expression of HSP70, for example, after exposure of cells to the class of HSP90 inhibitory therapeutic agents called geldanamycins.¹³ In Park et al, we note that OSU-03012 exposure suppresses HSP90 expression concomitantly with increasing HSP70 levels; thus, OSU-03012 may in addition to causing an ER stress and autophagy response, be an HSP90 inhibitor, albeit a relatively weak one. If this is the case, additional exploration and chemical-synthetic modification of OSU-03012 into a potent HSP90 inhibitor would be warranted. Furthermore, in Park et al. we

also note that geldanamycin-induced HSP70 expression is significantly enhanced in PERK^{-/-} cells, which correlates with abolition of drug-induced cell killing. These findings strongly argue that PERK signaling plays a central role in the reciprocal regulation of HSP90 and HSP70 expression in transformed cells and argue that ER stress and autophagy are linked into heat shock protein biology and to transformed cell survival. They also argue that Celecoxib-like drugs in a COX2-independent fashion may have one component of their biology which acts to modulate expression of heat shock proteins, the levels of ER stress and of autophagy.

Studies from our laboratory and that of Dr. Steven Grant are some of the first using novel cancer therapeutic agents in combination to demonstrate that when exposed to moderately toxic concentrations of a single therapeutic agent, cancer cells can exhibit rapid short-term compensatory survival responses by activating multiple survival signaling pathways or by increasing the expression of certain proteins that maintain cell viability that can, in turn, be blocked to cause profound levels of killing by parallel administration of a second therapeutic agent.¹⁴⁻¹⁸ These compensatory signaling responses occur prior to any “long term” adaptive changes, such as the development of mutations which would, as it were, “lock in” a profound level of cellular resistance to any single therapeutic drug, e.g., as has been observed for some tyrosine kinase inhibitors. “Classical” ER stress signaling is also noted to either rapidly promote a toxic response, e.g., elevated CHOP expression, or rapidly promote a protective response e.g., enhanced BiP/Grp78 expression, based on the duration or intensity of the ER stress signal.¹⁹

Celecoxib-induced apoptosis is argued to be ER stress-dependent, with loss of GADD153 (CHOP) function preventing cell killing.^{20,21} In this model, simplistically, Celecoxib-induced apoptosis would be impeded in cells lacking PERK expression, which is the opposite of our findings with OSU-03012 treatment, wherein transformed fibroblasts are more resistant to drug toxicity when PERK is not expressed. Indeed, parallel studies in wild type and PERK^{-/-} cells using OSU-03012 and thapsigargin demonstrate diametrically opposite effects in drug sensitivity. To our surprise we find that knockdown of CHOP expression modestly reduces OSU-03012 toxicity in transformed MEFs but has no effect on survival in HCT116 colorectal or U251 cells. OSU-03012 decreases Grp78/BiP expression and has no effect on CHOP expression, whereas in a “classical” ER stress response the expression of these proteins is expected to rise.^{22,23}

Recently, we note that the novel clinically relevant drug Sorafenib, which has multiple intracellular targets, including Raf family kinases and receptor tyrosine kinases, and based on the structure of the Raf-1 tyrosine kinase domain the probability of Src-like family kinases also, causes an ER stress response that also correlates with a reduction in the expression of Grp78/BiP, suggesting that the response pattern we observe with OSU-03012 may be a characteristic of drugs which inhibit both, to use a generic term, “signaling pathways,” and that also cause ER stress responses.¹⁹ In other words, although a “true” hypothesis-driven scientific approach has lead drug companies to develop agents that are supposedly highly specific and targeted towards a single specific molecule, e.g., ERBB family tyrosine kinase inhibitors; this approach may not be beneficial to the translation of drugs as single agents into the clinic. Indeed, although exceptions exist such as Gleevec, many drugs that appear to specifically inhibit only one target with a low nM or pM IC₅₀ in a tumor cell in vitro

are often not effective in vivo at generation of a measurable therapeutic effect, possibly because tumor cells are only partially addicted to any one cell survival pathway and can rapidly switch their survival addiction between different pathways prior to mutational resistance development. Of additional note with respect to the actions of OSU-03012, some agents, e.g., the proteasome inhibitor Velcade, have been shown to kill tumor cells via increased expression of ER stress markers such as CHOP while inhibiting PERK activity and the phosphorylation of eIF2 α .²⁴ Thus, agents such as Sorafenib and OSU-03012, which inhibit multiple kinase proteins within signaling pathways, and also elicit useful toxic responses such as ER stress and autophagy, may represent “switch hitters,” being able to hit on many sides of the plate to score a home run and kill a cancer cell.

In conclusion, studies in Park et al demonstrate that the regulation of transformed cell survival following treatment of cells with low doses of OSU-03012 is complicated and multi-factorial. OSU-03012 activates PERK which: (a) through a yet to be identified eIF2 α -independent mechanism stimulates autophagic vacuolization which plays a role in the activation of cathepsin proteases; (b) induces caspase 4- and cathepsin-dependent complete vacuolization of LC3-containing vesicles into LysoTracker red-staining low pH vesicles and a subsequent enhancement in cell killing via BID cleavage; and (c) promotes increased expression of HSP70 that acts to suppress cell killing potentially by blocking early vacuolization processes and the lethal actions of AIF.²⁵

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References

- Hawkey CJ, Fortun PJ. Cyclooxygenase-2 inhibitors. *Curr Opin Gastroenterol* 2005; 21:660-4.
- Klenke FM, Gebhard MM, Ewerbeck V, Abdollahi A, Huber PE, Skell A. The selective Cox-2 inhibitor Celecoxib suppresses angiogenesis and growth of secondary bone tumors: An intravital microscopy study in mice. *BMC Cancer* 2006; 12:6-9.
- Koehne CH, Dubois RN. COX-2 inhibition and colorectal cancer. *Semin Oncol* 2004; 31:12-21.
- Zhu J, Huang JW, Tseng PH, Yang YT, Fowble JW, Shiau CW, Shaw YJ, Kulp SK, Chen CS. From the cyclooxygenase-2 inhibitor celecoxib to a novel class of 3-phosphoinositide-dependent protein kinase-1 inhibitors. *Cancer Res* 2004; 64:4309-18.
- Yacoub A, Park MA, Hanna D, Hong Y, Mitchell C, Pandya AP, Harada H, Powis G, Chen CS, Koumenis C, Grant S, Dent P. OSU-03012 promotes caspase-independent but PERK-, cathepsin B-, BID-, and AIF-dependent killing of transformed cells. *Mol Pharmacol* 2006; 70:589-603.
- Johnson AJ, Smith LL, Zhu J, Heerema NA, Jefferson S, Mone A, Grever M, Chen CS, Byrd JC. A novel celecoxib derivative, OSU03012, induces cytotoxicity in primary CLL cells and transformed B-cell lymphoma cell line via a caspase- and Bel-2-independent mechanism. *Blood* 2005; 105:2504-9.
- Tseng PH, Lin HP, Zhu J, Chen KF, Hade EM, Young DC, Byrd JC, Grever M, Johnson K, Druker BJ, Chen CS. Synergistic interactions between imatinib mesylate and the novel phosphoinositide-dependent kinase-1 inhibitor OSU-03012 in overcoming imatinib mesylate resistance. *Blood* 2005; 105:4021-7.
- Tseng PH, Wang YC, Weng SC, Weng JR, Chen CS, Brueggemeier RW, Shapiro CL, Chen CY, Dunn SE, Pollak M, Chen CS. Overcoming trastuzumab resistance in HER2-overexpressing breast cancer cells by using a novel celecoxib-derived phosphoinositide-dependent kinase-1 inhibitor. *Mol Pharmacol* 2006; 70:1534-41.
- To K, Zhao Y, Jiang H, Hu K, Wang M, Wu J, Lee C, Yokom DW, Stratford AL, Klinge U, Mertens PR, Chen CS, Bally M, Yapp D, Dunn SE. The phosphoinositide-dependent kinase-1 inhibitor, OSU-03012, prevents Y-box binding protein-1 (YB-1) from inducing epidermal growth factor receptor (EGFR). *Mol Pharmacol* 2007; 72:641-52.

10. Caron RW, Yacoub A, Li M, Zhu X, Mitchell C, Hong Y, Hawkins W, Sasazuki T, Shirasawa S, Kozikowski AP, Dennis PA, Hagan MP, Grant S, Dent P. Activated forms of H-RAS and K-RAS differentially regulate membrane association of PI3K, PDK-1, and AKT and the effect of therapeutic kinase inhibitors on cell survival. *Mol Cancer Ther* 2005; 4:257-70.
11. Park M, Yacoub A, Rahmani M, Zhang G, Hart L, Hagan M, Calderwood S, Sherman M, Koumenis C, Spiegel S, Chen CS, Graf M, Curiel D, Fisher P, Grant S, Dent P. OSU-03012 stimulates PERK-dependent increases in HSP70 expression, attenuating its lethal actions in transformed cells. *Mol Pharmacol* 2008, [Epub ahead of print].
12. White VL, Johnson A, Chen CS, Farag SS. OSU-03012, a novel celecoxib derivative, is cytotoxic to myeloma cells and acts through multiple mechanisms. *Clin Cancer Res* 2007; 13:4750-8.
13. Mitchell C, Park MA, Zhang G, Han SI, Harada H, Franklin RA, Yacoub A, Li PL, Hylemon PB, Grant S, Dent P. 17-Allylamino-17-demethoxygeldanamycin enhances the lethality of deoxycholic acid in primary rodent hepatocytes and established cell lines. *Mol Cancer Ther* 2007; 6:618-32.
14. Dent P, Yacoub A, Fisher PB, Hagan MP, Grant S. MAPK pathways in radiation responses. *Oncogene* 2003; 22:5885-96.
15. Valerie K, Yacoub A, Hagan MP, Curiel DT, Fisher PB, Grant S, Dent P. Radiation-induced cell signaling: Inside-out and outside-in. *Mol Cancer Ther* 2007; 6:789-801.
16. Grant S, Dent P. Kinase inhibitors and cytotoxic drug resistance. *Clin Cancer Res* 2004; 10:2205-7.
17. McKinstry R, Qiao L, Yacoub A, Dai Y, Decker R, Holt S, Hagan MP, Grant S, Dent P. Inhibitors of MEK1/2 interact with UCN-01 to induce apoptosis and reduce colony formation in mammary and prostate carcinoma cells. *Cancer Biol Ther* 2002; 1:243-53.
18. Hawkins W, Mitchell C, McKinstry R, Gilfor D, Starkey J, Dai Y, Dawson K, Ramakrishnan V, Roberts JD, Yacoub A, Grant S, Dent P. Transient exposure of mammary tumors to PD184352 and UCN-01 causes tumor cell death in vivo and prolonged suppression of tumor regrowth. *Cancer Biol Ther* 2005; 4:1275-84.
19. Rahmani M, Davis EM, Crabtree TR, Habibi JR, Nguyen TK, Dent P, Grant S. The kinase inhibitor sorafenib induces cell death through a process involving induction of ER stress. *Mol Cell Biol* 2007; 27:5499-513.
20. Tsutsumi S, Gotoh T, Tomisato W, Mimo S, Hoshino T, Hwang HJ, Takenaka H, Tsuchiya T, Mori M, Mizushima T. Endoplasmic reticulum stress response is involved in nonsteroidal anti-inflammatory drug-induced apoptosis. *Cell Death Differ* 2004; 11:1009-16.
21. Tsutsumi S, Namba T, Tanaka KI, Arai Y, Ishihara T, Aburaya M, Mima S, Hoshino T, Mizushima T. Celecoxib upregulates endoplasmic reticulum chaperones that inhibit celecoxib-induced apoptosis in human gastric cells. *Oncogene* 2006; 25:1018-29.
22. Ron D. Translational control in the endoplasmic reticulum stress response. *J Clin Invest* 2002; 110:1383-98.
23. Rutkowski DT, Kaufman RJ. A trip to the ER: Coping with stress. *Trends Cell Biol* 2004; 14:20-8.
24. Nawrocki ST, Carew JS, Dunner Jr K, Boise LH, Chiao PJ, Huang P, Abbruzzese JL, McConkey DJ. Bortezomib inhibits PKR-like endoplasmic reticulum (ER) kinase and induces apoptosis via ER stress in human pancreatic cancer cells. *Cancer Res* 2005; 65:11510-9.
25. Nylandsted J, Gyrd-Hansen M, Danielewicz A, Fehrenbacher N, Lademann U, Hoyer-Hansen M, Weber E, Multhoff G, Rohde M, Jäättelä M. Heat shock protein 70 promotes cell survival by inhibiting lysosomal membrane permeabilization. *J Exp Med* 2004; 200:425-35.