Author's response to reviews

Title: The Aryl Hydrocarbon Receptor Ligand Omeprazole Inhibits Breast Cancer Cell Invasion and Metastasis

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Version: 2 Date: 16 May 2014

Author's response to reviews: see over
Reviewer 1

Concern 1: The authors refer to their previous papers and to the results summarized in Table 1, which showed a dualism of AHR-active pharmaceuticals which can exhibit of AHR agonist or AHR-antagonist activities dependent on the cell context. In addition, omeprazole sulphide appears to be a ligand and antagonist of the mouse AHR (Gerbal-Chaloin et al., Cell Signal. 2006;18(5):740-50). However, there is no discussion trying to explain these effects. Therefore, the conclusion that omeprazole may be used for breast cancer chemotherapy can be a bit speculative.

Ligands for the AhR and other receptors exhibit tissue-specific agonist/antagonist activities and are select receptor modulators (SRMs). We have recently discussed SRMs in two recent review articles (Toxicol. Sci. 135, 1, 2013; Mol. Endocrinol. 28, 157, 2014) and this concept is now amplified in this manuscript.

Concern 2: Induction of Cyp1a1 is a nonspecific biomarker of aryl hydrocarbon receptor activation (Hu et al., Mol Pharmacol. 2007 Jun;71(6):1475-86). Did the authors analyze other AHR-regulated genes such as Cyp1a2, LY6E, Ugt1a1, Nqo1?

Induction of Cyp1a1 mRNA is the most widely used biomarker of Ah-responsiveness; however, we have also included results from the induction of CYP1A1 protein (Suppl. Fig. 3) and CYP1B1 mRNA (Suppl. Fig. 2).

Concern 3: The authors stated that omeprazole-dependent inhibition of breast cancer cell invasion depends on AHR-mediated down regulation of CXCR4 expression. However, the RNA interference experiments showed in Figures 5A and 5C do not include control scrambled) siRNA, and therefore cannot be conclusive.

All of the untreated (-) lanes in Figure 5A and 5C are transfected with a scramble (control) siRNA and this is now noted in the Methods section.

Concern 4: Omeprazole is known to regulate genes expression through the different mechanisms. For example, omeprazole blocks STAT6 binding to the eotaxin-3 promoter (Zhang et al., PLoS One. 2012;7(11):e50037). It would be helpful if the authors will be able to check if the omeprazole-dependent regulation of CXCR4 expression can be abrogated by disruption of the DRE sequences within CXCR4 promoter.
Figures 5C and 6A containing 4 DRE sequences including 3 that overlap and a detailed mutation analysis of individual and multiple DREs is beyond the scope of this paper. However, our ChIP assays clearly demonstrate ligand (omeprazole)-dependent recruitment of the AhR to both DRE-123 (overlapping) and DRE-4.

**Concern 5:** Figure 5B: there is no dose-dependent effect of omeprazole concentration of CXCR4 promoter binding by AHR. Please explain.

This paper shows that for omeprazole, there is a dose-dependent effect on inhibition of cell invasion (Figs. 1 and 2A), migration (Fig. 3C), CYP1A1 mRNA (Fig. 4A), and AhR-DRE binding (Fig. 6B). The promoter studies (Fig. 5C) were used as a model for determining the effects of AhR antagonists on omeprazole-mediated downregulation of CXCR4 and, therefore, only one concentration of omeprazole was used.

**Reviewer 2**

*This study is original and well defined. The experimental work presented is well designed with appropriate controls and the conclusions of the paper are strongly supported by the data. The main limitation of this study is that the high doses of omeprazole may not be attainable in patients and there is no toxicity information presented on how the mice fared with the high dosing of omeprazole. What was the MTD of omeprazole in the mice? Were there pharmacokinetic measurements of omeprazole to support the conclusions that omeprazole is inhibiting tumor metastasis?*

As indicated by the Reviewer, this is an "original" study and as pointed out by the Reviewer, the "high doses" may not be attainable and this issue is discussed at the end of our Discussion. Pharmacokinetic studies in mouse models were not carried out and are beyond the scope of this study. We hope that clinicians will use and extend our results in patients with breast cancer metastasis using omeprazole or possibly other more effective structural analogs.

**Major concerns:**

1. The role of FAK in omeprazole-mediated inhibition of cancer cell migration isn't addressed, but previously AHR has been demonstrated to inhibit HUVEC motility, in part through inhibition of FAK [Cell Mol Life Sci. 2009 Oct; 66(19):3193-205]. Also, CXCR4 promotes FAK and MMP9 expression. Omeprazole inhibition of CXCR4 gene expression could reduce FAK expression and activity, explaining more directly how omeprazole inhibits cancer cell motility.

We have not looked extensively for effects downstream from CXCR4 [except for MMP-9 (Fig. 5B)] but this will be examined in future studies.
2. The role of endothelial cell migration and angiogenesis should be addressed. The lack of metastatic growth in the tail vein metastasis experiment could be related to inhibition of angiogenesis as well as cell-intrinsic inhibition of cancer cell migration. CD31 and VWF staining of the tissue sections may help clarify this issue.

The focus of our in vivo experiments was to complement the in vitro studies and we showed that omeprazole inhibited both CXCR4 and PCNA expression in lung metastasis (Fig. 3). We plan to carry out future experiment on inhibition of angiogenesis and metastasis by omeprazole in transgenic models; however, this is beyond the scope of this study.

Minor concerns:

1. Figure 2B is labeled as testing 3',4'-methoxy-alpha-nitroflavone and should be listed as testing 3',4'-methoxy-alpha-naphthoflavone.

2. The discussion repeats some information in the results section and may be shortened.

This mislabeling (Fig. 2B) has been corrected and some redundancies in the Discussion have been deleted.

Reviewer 3

(a) Throughout the manuscript, there is a lack of clarity in distinguishing between those distinct states of AhR and their defined roles in cancer invasion and metastasis.

We now make it clear that AhR agonists inhibit growth and/or metastasis of ER-positive and ER-negative breast cancer cells.

(b) If the underlying assumption for Omeprazole agonistic effect is the activation of AhR, it is troubling that previous report from this lab has shown that MCDF, an AhR antagonist (which by definition blocks AhR activation), was successful in inhibiting growth and metastasis of ER-negative breast cancer cell lines in animal models.

MCDF, like omeprazole, inhibits metastasis and growth of ER-negative breast cancer. MCDF is a selective AhR modulator (SAhRM) that exhibits tissue and response-specific antagonist and agonist activities (e.g. compared to TCDD). In breast cancer cells, MCDF acts as an AhR agonist. The concept and function of SAhRMs is now amplified in the revised manuscript.
(c) If the effect is through stabilization of AhR protein, it is hard to reconcile the presented data, and the rationale for the majority of experiments. For instance, data presented in Supplemental Fig. 3 showed that treatment with 10 nM TCDD for 24 h resulted in depletion of AhR by at least 80% (consistent with what is established in this field). This effect of TCDD on AhR was associated with a decrease in invasion (Figs. 1A & 1D). However, depletion of AhR by siAhR was shown in Fig. 2A to result in increased cellular invasion both under basal (DMSO) or TCDD- treatment. Further experiments and more discussion is needed to clarify this discrepancy.

Ligand for the AhR and other receptors such as the estrogen receptor (ER) may or may not induce proteasome-dependent degradation of their cognate receptor. The rationale for these ligand-dependent differences in receptor degradation and the functional consequences are not well understood and this is now pointed in the revised manuscript. Knockdown of the AhR by RNA interference blunts ligand-induced response due to low initial levels of the receptor which results in decreased AhR-responsiveness.

(d) Unlike TCDD, treatment of cells with omeprazole at all three doses resulted in stabilization of AhR protein (Supplemental Fig. 3). This is inconsistent with AhR-ligand signaling cascade, where the activation of AhR by its agonist results in its transformation to function as a transcription factor, followed by its nuclear export to the cytoplasm to be degraded by proteosomes. This lack of omeprazole-induced AhR down-regulation is accompanied by its very modest induction of the prototype AhR-responsive gene, CYP1A1 (Supplemental Fig. 1). No rationalization was proposed for this disparate response of omeprazole and its divergence from the defined AhR-agonist signaling pathways. However, when omeprazole exerted substantial inhibition of cellular invasion (50-70%), while TCDD, the far more potent AhR agonist, inhibited invasion only by <20% (Figs. 1A, 1C & 1D), the authors related omeprazole effect to its AhR agonistic activity. In spite of this weak ground for AhR agonistic response of omeprazole, the majority of the presented experiments and resulting data were set out to prove that, using different AhR antagonists and AhR siRNA, which is further confusing given the previous report from the group using other antagonists.

This comment is similar to that in (c). Although receptor levels observed after 24 hr treatment with TCDD and omeprazole are low and high, respectively, the potency of these compounds as CYP1A1 inducers is reversed! Thus, receptor levels after treatment do not predict the efficacy or potency of an AhR ligand for a specific response and this is now pointed out in the revised manuscript. For example, in non-tumor tissue, TCDD invariably is the most potent AhR agonist and AhR levels are dramatically decreased in target organs/tissues but presumably are restored (and again degraded) at later time points.

As correctly stated by the authors on page 10 (2nd line), the induction of CYP1A1 is a prototypical readout for AhR activation by its ligands (agonists). From receptor pharmacology point of view, it is hard to put in perspective that an effect through this
receptor will require a minimum concentration of omeprazole (200 $\mu$M), which is $5 \times 10^5$ higher than TCDD concentration, that was sufficient to induce a 10-fold higher level of CYP1A1 mRNA (Supplemental Fig. 1). It might have helped a little if doses of omeprazole were used in combination with TCDD, to establish whether they function additively or competitively.

These observations are typical for weak AhR agonists such as omeprazole and maximal induction (as observed for TCDD) may not be observed due to cytotoxicity. However, in the absence of cytotoxicity, some weak AhR ligands such as tryptamine induce CYP1A1 mRNA levels similar to that observed for TCDD (e.g. our recent paper in Mol. Pharmacol. 85, 877, 2014) but this is observed at concentrations of tryptamine $>10^3$ times higher than TCDD.

Authors could have entertained other possible mechanism(s) for omeprazole. For example, an effect of omeprazole on inhibiting proliferation and inducing apoptosis in pancreatic cancer cell lines was postulated to result from its interaction with the regulatory functions of the vATPase without inhibiting its pump function. (Udelnow et al. 2011. Omeprazole Inhibits Proliferation and Modulates Autophagy in Pancreatic Cancer Cells. PLoS ONE 6(5): e20143. doi:10.1371/journal.pone.0020143).

In this paper, we show that the effects of omeprazole on breast cancer cell migration, invasion, CYP1A1 induction, CXCR4 inhibition, and recruitment of the AhR to "DREs" are all attenuated by AhR knockdown or antagonists. Thus, we conclude that the effects of omeprazole are mediated by the AhR; however, this does not exclude other contributory pathways and this is now noted in the Discussion.