The Induction of Phase 2 Response in Chemoprevention by Heteraromatic Quinols

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ABSTRACT
Cancer chemoprevention has gained more and more attention over the past 25 years and has been successful in many in vitro and in vivo studies and also human intervention trials. Electrophiles from the metabolic activation of chemical carcinogens and reactive oxygen species are reactive intermediates that play a significant role in carcinogenesis. Cancer chemoprevention counteracts these reactive intermediates by the induction of phase 2 response. The induction of phase 2 response is mediated by the transcription factor Nuclear factor-erythroid 2-related factor 2 (Nrf2) which regulates phase 2 genes by binding to the antioxidant response element (ARE) in their promoters. Quinol analogues are promising chemopreventive agents that induce phase 2 enzymes via activation of Nrf2. This is due to the presence of Michael reaction acceptors group in the chemical structure of the arylquinol compounds. In this study, the quinol analogues were shown to increase Nrf2 protein level, PMX290 and BW114 being the most effective while PMX464 increased Nrf2 transcriptional activation markedly compared the other quinols. The data were validated by Western blot analysis and ARE reporter assays. This study expands the horizon of cancer chemoprevention and stresses the importance of Nrf2 in the defense against carcinogens and toxins.

INTRODUCTION
Over the past 25 years, chemoprevention in cancer has been successful in many in vitro and in vivo studies and has also been validated in human intervention trials (Sporn MB 2000 and 2002). Cancer chemoprevention counteracts the electrophiles formed during metabolic activation of chemical carcinogens and reactive oxygen species (ROS) generated from endogenous and exogenous sources by induction of phase 2 proteins. Chemopreventive agents induce phase 2 detoxifying enzymes and antioxidant enzymes (e.g. glutathione S-transferase, NAD(P)H quinine oxidoreductase 1 and heme oxygenase-1). This process serves as a major protective mechanism against carcinogens and is mediated by the transcription factor Nuclear factor-erythroid 2-related factor 2 (Nrf2) (Giudice and Montella 2006). Under normal conditions, Keap1 anchors Nrf2 and targets it for ubiquitination and consequently 26S proteosome-mediated degradation. This represses the ability of Nrf2 to induce phase 2 genes. Upon exposure to chemopreventive agents and oxidative stress, ubiquitination of Nrf2 by Keap1 is inhibited. Consequently, Nrf2 accumulates and translocates into the nucleus. In the nucleus, the transcription factor Nrf2 binds to the antioxidant response elements (ARE) within the promoter region of phase 2 genes. The binding of Nuclear factor-erythroid 2-related factor 2 (Nrf2) to the response...
elements (ARE) promotes the transcription of phase 2 detoxifying enzymes and antioxidant enzymes.

The main objective of this project is to investigate the induction of the phase 2 response by heteroaromatic 4-arylquinolins. The quinol compounds used in this project are PMX464, PMX290 and BW114. This compound class demonstrates antiproliferative activity in vitro and antitumor activity in vivo in tumor xenografts and is currently being developed for use in preclinical studies and clinical trials (Chew et al 2008). The chemical structure of the arylquinol compounds enable them to react readily with nucleophiles (due to their Michael reaction acceptors group). Therefore, the arylquinol compounds are likely to bind to various other cellular target proteins with accessible cysteine residues that have a low pKa and therefore highly reactive. This interaction is suggested to contribute to their anticancer effects (Bradshaw 2005).

It is hypothesised that quinol compounds act as chemopreventive agents by chemically modifying specific cysteine residues on Keap1. This causes the dissociation of NRF2 from the Keap1 and the inhibition of NRF2 ubiquitination and proteasome dependent degradation. This results in the accumulation of free NRF2 that can translocate to the nucleus to induce phase 2 enzyme transcription. This project aims to investigate the mechanism of various quinol analogues in the induction of phase 2 response. The understanding of the mechanism will help in the discovery of novel drugs in chemoprevention in future cancer research. To this end, I have investigated the effects of quinol analogues on the Nrf2 protein level and transcriptional activation to induce phase 2 response.

MATERIALS AND METHODS

The quinol analogues PMX464, PMX290 and BW114 were obtained from Pharminox, Nottingham, United Kingdom). Methods used in the experiment include cell culture and transfection techniques, plasmid construct of Nrf2-HA, truncated Nrf2-HA and Keap1-FLAG, immunoblotting, immunoprecipitation and ARE luciferase reporter assay.

RESULTS AND DISCUSSION

Quinol analogues increase Nrf2 protein level to induce phase 2 response.

Since the induction of phase 2 detoxifying enzymes and antioxidant enzymes is mediated by Nrf2 level, it can be suggested that an increase in Nrf2 protein level would induce an increase in phase 2 response. It was found that upon treatment with quinol analogues, Nrf2 protein level in the cell increased. From Figure 1, PMX290 and BW114 showed a marked increase in Nrf2 protein level compared to untreated cells. PMX464 showed a slight increase in Nrf2 protein level. It is suggested that the quinol analogues PMX464, PMX290 and BW114 prevent the ubiquitination of Nrf2, thus increasing its protein level. An interesting point to note is that 2µM of these two drugs were more potent than the positive control, 10µM sulforaphane as increase was more evident.

Quinol analogues increase Nrf2 transcriptional activation to induce phase 2 response.

Since Nrf2 regulates phase 2 response by binding to and transactivating antioxidant response elements (AREs) within the promoters of phase 2 target genes, the ARE gene
reporter assay was used to study the transcriptional activation of NRF2 (Giudice & Montella, 2006). HEK293 and HCT116 cells were transfected with plasmid containing ARE next to the gene encoding for luciferase. The binding and transactivating of Nrf2 to ARE will activate downstream transcriptional activity of luciferase. The luciferase expression can be detected by measuring luminescence using Steady-Glo Luciferase Assay. Hence, the binding and transactivation of Nrf2 to ARE can be quantified by measuring luciferase expression. The increase in transcriptional activation of Nrf2 would induce an increase in luminescence. From Figure 2, PMX464 induced the highest increase in Nrf2 transcriptional activation in treated cells compared to untreated cells, followed by PMX290 and then BW114. Because sulforaphane has been proven to prevent ubiquitination of Nrf2, it is used as a positive control to show the expected increase in Nrf2 transcriptional activation.

Figure 1. Nrf2 Protein level due to various treatments on HEK293 cells and HCT116 cells. (A) The effect of the drug treatment on transfected Nrf2-HA protein level in HEK293 cells. (B) The effect of the drug treatment on transfected Nrf2-HA protein level in HCT116 cells. (C) The effect of the drug treatment on endogenous Nrf2 protein level in HEK293 cells.

Figure 2. Data of one experiment that best represent the trend for fold increase of luminescence representing luciferase activity. The values represent the fold increase in luciferase activity compared to the untreated control.
Preventing Nrf2 ubiquitination by truncating Nrf2-Keap1 binding site and using dominant negative Ubc12 HEK293 cells.

Theoretically, an increase in Nrf2 protein level would increase Nrf2 transcriptional activity. However, PMX290 and BW114 induced the highest increase in Nrf2 protein level (Figure 1) while PMX464 induced the highest increase in Nrf2 transcriptional activity (Figure 2). This is surprising because we would expect the same quinol analogue to induce the highest increase in Nrf2 protein level and transcriptional activity. To investigate this, Nrf2 ubiquitination was prevented by truncating the Nrf2-Keap1 binding sites. If the increase in transcriptional activation by the drug is not due to the prevention of Nrf2 ubiquitination, there would be in an increase in transcriptional activation. Unfortunately, the results were inconclusive as the expression of truncated Nrf2 was too low and it was hard to distinguish the effects of truncated Nrf2 and endogenous Nrf2. Dominant negative Ubc12 HEK293 was then used to prevent Nrf2 ubiquitination. These results were inconclusive too.

Determination of the specific cysteine modification by the PMX290 using mass spectrometry.

To determine the sites in Keap1 modified by PMX290, HEK293 cells were transfected with plasmid containing Keap1 tagged with FLAG and after 2 days, lysed. 10 μM PMX290 treatment was done in vitro for 1 hour. PMX290 is expected to bind to Keap1 and the Keap1-PMX290 complex is isolated by immunoprecipitation using Anti-FLAG M2 agarose beads. The protein were then denatured in SDS sample buffer and subjected to SDS-PAGE and Western blotting. The gel was stained using Coomassie blue staining. Keap1 samples were excised and submitted for trypsin digestion and MALDI-TOF mass spectrometry analysis. The cysteine residues on Keap1 that have been implicated to be most likely modified are Cys-151, Cys-273 and Cys-288 (Kobayashi et al., 2009). The expected increase in m/z on peptides with cysteine residue is 383 but no such increase in m/z was seen.

REFERENCES


