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Screening natural products for inhibitors of quinone reductase-2 using ultrafiltration LC-MS

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Abstract

Inhibitors of quinone reductase-2 (NQO2; QR-2) can have anti-malarial activity and anti-tumor activities or can function as chemoprevention agents by preventing the metabolic activation of toxic quinones such as menadione. To expedite the search for new natural product inhibitors of QR-2, we developed a screening assay based on ultrafiltration liquid chromatography-mass spectrometry that is compatible with complex samples such as bacterial or botanical extracts. Human QR-2 was prepared recombinantly, and the known QR-2 inhibitor, resveratrol, was used as a positive control and as a competitive ligand to eliminate false positives. Ultrafiltration LC-MS screening of extracts of marine sediment bacteria resulted in the discovery of tetrangulol methyl ether as an inhibitor of QR-2. When applied to the screening of hop extracts from the botanical, *Humulus lupulus* L., xanthohumol and xanthohumol D were identified as ligands of QR-2. Inhibition of QR-2 by these ligands was confirmed using a functional enzyme assay. Furthermore, binding of xanthohumol and xanthohumol D to the active site of QR-2 were confirmed using X-ray crystallography. Ultrafiltration LC-MS was shown to be a useful assay for the discovery of inhibitors of QR-2 in complex matrices such as extracts of bacteria and botanicals.

INTRODUCTION

Quinone reductase-2 (NQO2; QR-2) is a cytosolic enzyme that is becoming a target for chemoprevention[1–3] due to several possible mechanisms of action including anti-malarial[4,5] and anti-tumor acitivities,[6–8] as well as preventing toxicity by certain quinones such as menadione.[9,10] An example of a natural product and dietary inhibitor of QR-2 is the cancer chemopreventive agent resveratrol which is abundant in grapes, nuts, and red wine.[6] New and more potent inhibitors of QR-2 are needed as chemoprevention agents, and the

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SUPPORTING INFORMATION AVAILABLE

X-ray structure determination of QR2-xanthohumol and QR2-xanthohumol D complexes. This material is available free of charge via the Internet at http://pubs.acs.org.

discovery of more natural product inhibitors like resveratrol might provide leads to these compounds.

Finding new inhibitors to macromolecular targets among complex extracts of botanicals and bacterial cultures requires a selective screening assay to reduce time, cost, and the incidence of false positives. To address these requirements, we have developed affinity mass spectrometry-based screening assays using ultrafiltration[11,12] and magnetic beads[13] to screen complex mixtures of potential ligands. When the macromolecular target is soluble such as a cytosolic protein, ultrafiltration liquid chromatography-mass spectrometry (LC-MS) screening is particularly useful because the receptor is maintained in solution during binding and screening. During ultrafiltration LC-MS, ligands in a mixture are allowed to bind to the target protein, ultrafiltration is used to separate the protein-ligand complexes from unbound low mass molecules, and then the retained ligands are released from the denatured receptor and analyzed using LC-MS. Examples include ultrafiltration LC-MS screening for ligands to the estrogen[14] and retinoid X receptors.[15]

To the best of our knowledge, no screening assay has been reported previously for the discovery of QR-2 ligands or inhibitors from complex mixtures such as extracts of marine organisms or botanicals. Since QR-2 is a cytosolic enzyme, the application of a solution-phase screening technique such as ultrafiltration LC-MS was appropriate to address the unmet need for QR-2 ligand discovery from complex matrices such as extracts of botanicals and marine sediment bacteria. Background noise due to non-specific binding of compounds to the ultrafiltration membrane was minimized by introducing a second membrane during the ligand-protein dissociation step. Characterization of each ligand using LC-MS and tandem mass spectrometry with high resolution accurate mass measurement facilitated structure determination. Binding to the active site of each new ligand was confirmed through competition with the known QR-2 inhibitor, resveratrol, and functional enzyme assays were carried out to determine the potency of each ligand as an inhibitor of QR-2. Finally, X-ray crystallography was used to confirm the binding of ligands within the active site of QR2 and to determine the geometry of their bound structures.

EXPERIMENTAL SECTION

Chemicals and reagents

All solvents were HPLC grade or better and were purchased from Fisher (Hanover Park, IL). *trans*-Resveratrol was purchased from Sigma-Aldrich (St. Louis, MO), and centrifugal ultrafiltration filters (Microcon YM-30 and YM-10) were purchased from Millipore (Bedford, MA). An extract was prepared from an *Actinomyces sp.* that had been cultured from marine sediment as described previously.[16] A hop extract from the botanical *Humulus lupulus* L. was prepared as described previously,[17] and recombinant human QR-2 was prepared using standard procedures as reported elsewhere.[18] Tetrangulol methyl ether was isolated as described previously using extraction followed by column chromatography.[19,20] Xanthohumol and its monooxygenated analogue, xanthohumol D, were also purified as described previously.[21]

Binding to QR-2 and ultrafiltration

For ultrafiltration LC-MS screening, 2 μg of a natural product extract or 0.5 μg of a pure compound was incubated with 12 μg of human recombinant QR-2 in 150 μL of a buffer (pH 7.5) consisting of 100 mM Tris, 10% glycerol, 50 mM KCl, and 1 mM EDTA at room temperature for 2 h. After incubation, each mixture or extract was filtered through a 10,000 Da molecular weight cut-off ultrafiltration membrane by centrifugation at 13,000*g* for 7 min at 4 °C. The QR-2–ligand complexes were washed three times with 150 μL aliquots of 50 mM ammonium acetate (pH 7.5) followed by another centrifugation at 13,000*g* for 7 min to

remove the unbound compounds. The washed QR-2/ligand solution was transferred to a new 10,000 Da molecular weight cut-off ultrafiltration centrifuge tube, and the ligands were dissociated from QR-2 using 400 μL of methanol. This two ultrafiltration membrane procedure eliminated background signals from compounds non-specifically bound to the membrane.[15] After centrifugation, the ultrafiltrate containing ligands was dried under a stream of nitrogen. After reconstitution in 50% aqueous methanol, the QR-2 ligands were characterized using LC-MS. Identical incubations using denatured QR-2 were used to control for non-specific binding; and LC-MS peaks that increased in area in the experiments relative to the control containing denatured QR-2 indicated specific binding. To confirm the binding of new ligands to the active site of QR-2, experiments were repeated containing 25 μM resveratrol. If resveratrol displaced the ligand from QR-2 as indicated by a reduced peak area during LC-MS analysis, then the ligand was determined to bind to the active site of the enzyme.

LC-MS and LC-MS/MS

A 20 μL aliquot of each reconstituted ultrafiltrate was analyzed using a Thermo Finnigan (San Jose, CA) LCQ Deca ion trap mass spectrometer and negative ion electrospray. The mass spectrometer was interfaced to a Surveyor (Thermo) HPLC system and a Waters (Milford, MA) Xterra MS C18 column (2.1 mm × 150 mm, 3.5 μm). For LC-MS analysis of ultrafiltrates containing QR-2 ligands, a 32 min linear gradient was used from 25 to 100% acetonitrile in 0.05% aqueous acetic acid, followed by isocratic 100% acetonitrile for 3 min. The flow rate was 160 μL/min, and the column was re-equilibrated at least 10 min between analyses. Mass spectra were acquired over the range *m/z* 150 to *m/z* 800 every 0.5 s. High resolution accurate mass measurements and tandem mass spectra of xanthohumol and xanthohumol D were acquired using a Shimadzu (Kyoto, Japan) ion trap-time-of-flight of hybrid mass spectrometer at a normalized collision energy for collision-induced dissociation of 40%, ion accumulation time of 10 msec and an activation q value of 0.210. The ion source parameters for tandem mass spectrometry included a capillary voltage of −3.5 kV, source block temperature 200 °C, curved desolvation line temperature 200 °C, and nebulizer gas flow of 1.5 L/min. External calibration using a solution of sodium trifluoroacetate provided mass accuracy within 5 ppm.

Steady-steady kinetic assay and IC_{50} value determination

The kinetic activity of QR-2 was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide (MTT) as a substrate in an assay format that we have described previously.[22] Briefly, the reduction of MTT to formazan by QR-2 and the second substrate, the reduced form of *N*-ribosyl-nicotinamide, was monitored at 612 nm using a SpectraMax Plus 384 UV/vis spectrophotometer thermostatted at 23 °C. The assay mixture contained 15 nM QR-2, 25 μM *N*-ribosyl-nicotinamide, 200 μM MTT, and a reaction buffer containing 100 mM NaCl, 50 mM Tris HCl, and 0.1% Triton X-100 for a final assay volume of 200 μL. The initial slopes of the reaction (ΔAbsorbance/ΔTime) were used to calculate the initial rates using a molar absorptivity of 11,300 $mM^{-1}cm^{-1}$ for MTT. The IC_{50} values were determined in 96-well plates using the assay outlined above by varying the inhibitor concentrations from 75 μg/mL to 300 μg/mL and then fitting the kinetic data to the following equation: $\%Inhibition = \%Inhibition_{max}/(1+[I]/IC_{50})$; where [I] is inhibitor concentration, and %inhibition is calculated from control rates in the absence of inhibitor. Data were fit using the enzyme kinetics module of SigmaPlot (SPSS; Chicago, IL), and IC_{50} and percent maximal inhibition values were determined.

Crystallization of QR-2

Human QR-2 was expressed and purified from 4L of *Escherichia coli* BL21(DE3) cells according to our previously described procedures.[18] Purified QR-2 was concentrated to 24

mg/mL, as determined using the Bio-RAD Protein Assay, and then crystallized using the hanging-drop, vapor diffusion method by adding 1 μL of purified QR-2 to 1 μL of reservoir solution which contained 12 μM flavin adenine dinucleotide (FAD), 5 mM dithiothreitol, 100 mM NaCl, 100 mM BisTris pH 6.4, and 1.7 M ammonium sulfate. Diffraction quality crystals of QR-2 grew within two weeks. Individual crystals (approximately 0.5 mm in length and 0.1 to 0.2 mm in width) were transferred to 10 μL of an inhibitor solution which was prepared with 9 μL of the original reservoir solution plus 1 μL of either pure xanthohumol (4 mg/mL in DMSO) or pure xanthohumol D (4 mg/mL in DMSO). The final inhibitor concentration was 0.4 mg/mL. The crystals were allowed to soak with inhibitor for 48 h to maximize the potential for ligand binding. Crystals were then retrieved from the soaking solution using a nylon loop, swiped through soaking solution supplemented with 30% glycerol, and finally flash-frozen in liquid nitrogen. Crystals were stored in a Dewar containing liquid nitrogen until X-ray data collection. Experimental details concerning the X-ray structure determination of QR-2-xanthohumol and QR-2-xanthohumol D complexes are described in Supporting Information available on-line.

RESULTS AND DISCUSSION

To verify that this new ultrafiltration LC-MS assay could detect QR-2 ligands, the known ligand resveratrol was screened, and the results are shown in Figure 1. Using denatured QR-2 as a negative control, LC-MS analysis of the ultrafiltrates after binding, washing, and release of resveratrol from QR-2 indicated that resveratrol bound to active QR-2 but not to denatured QR-2. Resveratrol was detected as a deprotonated molecule of *m/z* 227 with a retention time of 10.7 min (Figure 1). No non-specific binding of resveratrol to QR-2 or the apparatus was detected as indicated by the lack of a resveratrol peak in the control analysis.

After confirming that ultrafiltration LC-MS screening may be used to detect ligands of QR-2 such as resveratrol, this approach was used to screen a series of extracts of marine sediment bacterial cultures. As shown in the ultrafiltration LC-MS chromatograms in Figure 2, a hit was detected in an extract of an *Actinomyces sp.* eluting at 29.7 min with a deprotonated molecule of *m/z* 317. Enhancement of the peak more than 5-fold compared to the control containing denatured enzyme indicated specific binding of the ligand to QR-2. The active compound was isolated and its structure was determined by 2D-NMR spectroscopy to be tetrangulol methyl ether (see structure in Figure 2). The ability of tetrangulol methyl ether to inhibit the action of QR-2 was ascertained by using an enzymatic inhibition assay which indicated that the IC_{50} value of tetrangulol methyl ether toward QR-2 is 0.16 μM. This functional inhibition assay provided confirmation of the initial screening results and evidence that tetrangulol methyl ether is an inhibitor of QR-2. Note that ultrafiltration LC-MS screening facilitates the identification of ligands to a macromolecular receptor but does not determine how well a ligand might function as an inhibitor.

Using the functional assay to prescreen botanical extracts, an ethanolic extract of hops (*Humulus lupulus* L.) was identified that showed an IC_{50} value for QR-2 of 28 μg/mL. The utility of ultrafiltration LC-MS for the screening of botanical extracts for ligands of QR-2 was then evaluated using this hop extract. As shown in Figure 3, an abundant QR-2 ligand was detected in the hop extract with a deprotonated molecule of *m/z* 353 and an LC-MS retention time of 27.6 min. Several compounds with deprotonated molecules of *m/z* 369 were detected at low abundance as QR-2 ligands with the most abundant eluting at a retention time of 22.8 min (Figure 3). To verify that these ligands were bound to the active site of QR-2 and were not false positive results due to non-specific binding, resveratrol was added to the incubation mixture as a competitive inhibitor, and the ultrafiltration LC-MS screening process was repeated. As shown in the computer-reconstructed mass chromatograms in Figure 4, the signals of all compounds of *m/z* 369 and the ligand of *m/z*

353 were attenuated or eliminated in the presence of 25 μM of the high affinity QR-2 ligand resveratrol. These results confirmed that these hop compounds bind to the active site of QR-2.

Based on co-elution with an authentic standard during LC-MS, identical deprotonated masses of *m/z* 353 and identical tandem mass spectra, the QR-2 ligand eluting at 27.6 min was identified as xanthohumol. Among the QR-2 ligands of *m/z* 369, the compound eluting at 22.8 min was identified as xanthohumol D based on comparison with an authentic standard. The negative ion tandem mass spectra of xanthohumol and xanthohumol D are shown in Figure 5. Xanthohumol and xanthohumol D were assayed for inhibition of QR-2 using a functional assay, and the IC_{50} values were determined to be 196 ± 68 μM and 110 ± 27 μM, respectively.

Although effective, competition with the high affinity ligand resveratrol is an indirect method to demonstrate that xanthohumol and xanthohumol D bind to the active site of QR-2. As a direct method to verify binding the active site, X-ray crystallographic analysis was used to visualize the binding of xanthohumol and xanthohumol D to the ligand binding pocket of QR-2. X-ray data were collected on the QR2-xanthohumol and QR2-xanthohumol D complexes to a resolution of 1.5 Å. X-ray data collection and refinement statistics are summarized in Table 1 of the Supporting Information. As shown in the XRD analysis in Figure 6, the electron density for xanthohumol D is clearly observed, and the inhibitor occupies the QR-2 binding pocket. Xanthohumol occupied the same site (data not shown). Both xanthohumol D and xanthohumol interact directly with the FAD cofactor in parallel orientations. It should be noted that crystalline structures of the QR-2 active site containing other bound inhibitors have been reported previously.[3,8,18] This XRD analysis validated the ultrafiltration LC-MS competition binding assay as a means of determining that QR-2 ligands bind to the enzyme active site.

The microbial natural product tetrangulol methyl ether was first reported by Kuntsmann and Mitscher in 1966[23] and is known to exhibit antibiotic properties.[24] The inhibition of QR-2 by tetrangulol methyl ether also suggests that it may have chemoprevention activity. Hops have long been used to flavor beer and also are used a dietary supplement for mood, sleep and relief of menopausal symptoms.[25,26] Xanthohumol has been reported to have chemoprevention activity through the alkylation of Keap1 and the Nrf2-related up-regulation of the antioxidant response element.[27,28] Inhibition of QR-2 by xanthohumol and xanthohumol D from the hops extract is another potential mechanism of chemoprevention for these compounds.

Compared with resveratrol (IC_{50} 5.1 μM[4]), xanthohumol and xanthohumol D are weaker inhibitors of QR-2 (IC_{50} >100 μM), but tetrangulol methyl ether is considerably more potent (IC_{50} 0.16 μM). Tetrangulol methyl ether inhibits QR-2 more effectively than melatonin which has an IC_{50} value of 11.3 μM,[18] 45 synthetic resveratrol analogs prepared by Sun *et al.*[2] (IC_{50} values ≥ 0.27 μM), and 197 flavonoids tested by Boutin *et al.*[29] (IC_{50} values ≥ 0.30 μM). The therapeutic agent imatinib is currently among the few compounds that can inhibit QR-2 more effectively than tetrangulol methyl ether. Used to treat leukemia, imatinib inhibits QR-2 with an IC_{50} value of 0.08 μM.[30] Among the natural products tested to date, tetrangulol methyl ether is perhaps the most potent inhibitor of QR-2.

CONCLUSIONS

A new assay based on ultrafiltration LC-MS and LC-MS/MS with high resolution accurate mass measurement has been developed for the identification of ligands to QR-2. Specific binding to the active site of QR-2 may be determined by repeating the ultrafiltration LC-MS

screening process in the presence of a high affinity ligand such as resveratrol. In combination with a functional assay, this approach may be used to identify inhibitors of QR-2 in complex mixtures of natural products. Specifically, ultrafiltration LC-MS screening was able to detect the QR-2 inhibitors xanthohumol and xanthohumol D in an extract of hops, as well as tetrangulol methyl ether in a culture of a marine sediment *Actinomyces sp*. No matrix interference was encountered.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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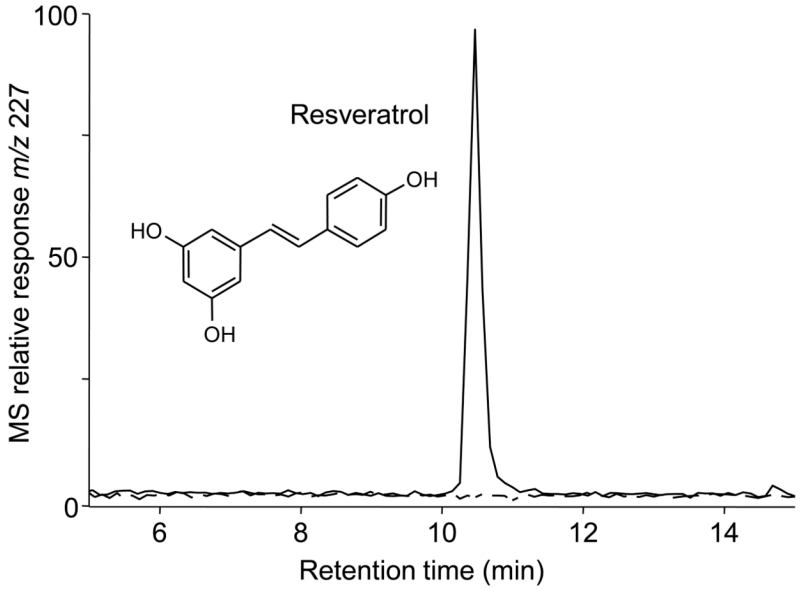

Figure 1.

Ultrafiltration LC-MS screening of resveratrol (2.5 μg/mL) for binding to QR-2 (80 μg/mL). LC-MS chromatograms of ultrafiltrates containing resveratrol released from active QR-2 (solid line) or denatured QR-2 (dashed line) as a control. Enhancement of the resveratrol peak (detected at *m/z* 227 as the deprotonated molecule during negative ion electrospray) compared to the control indicates specific binding to QR-2.

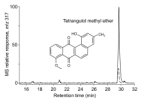

Figure 2.
Ultrafiltration LC-MS detection of tetrangulol methyl ester as a ligand of QR-2 (80 μg/mL) in an extract of an *Actinomyces sp.* (2 μg/mL) from marine sediment. Computer-reconstructed negative ion electrospray mass chromatograms of the signals of *m/z* 317 using negative ion electrospray are shown for the ultrafiltrates obtained with active QR-2 (solid line) or denatured QR-2 as a control (dashed line).

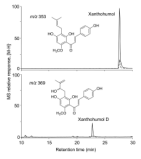

Figure 3.
Screening of an ethanolic hop extract (2 μg/mL) for ligands to QR-2 (80 μg/mL) by using ultrafiltration LC-MS. Compared to the control, which contained denatured enzyme (dashed line), LC-MS with negative ion electrospray LC-MS showed several peaks that were enhanced due to specific binding to QR-2 (solid line). A) The deprotonated molecule of xanthohumol at *m/z* 353 was detected at a retention time of 27.6 min; and B) isomeric compounds of *m/z* 369 containing one more oxygen than xanthohumol were detected including xanthohumol D eluting at 22.8 min. Identification of xanthohumol and xanthohumol D was based on comparison with authentic standards.

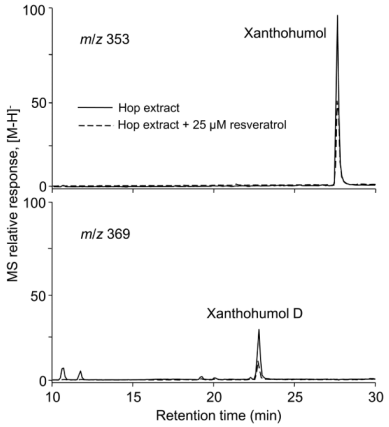

Figure 4.

Ultrafiltration LC-MS analysis of a hop extract as shown in Figure 3 (solid line), except that 25 μM of resveratrol was included in a competition experiment (dashed line). Resveratrol, a high affinity ligand added at high concentration, displaced xanthohumol and xanthohumol D from QR-2 indicating that all three compounds compete for the same binding site.

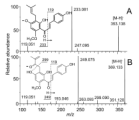

Figure 5.
Negative ion electrospray tandem mass spectra of the deprotonated molecules of A) xanthohumol at *m/z* 353; and B) xanthohumol D at *m/z* 369. Product ion mass spectra were obtained using collision-induced dissociation.

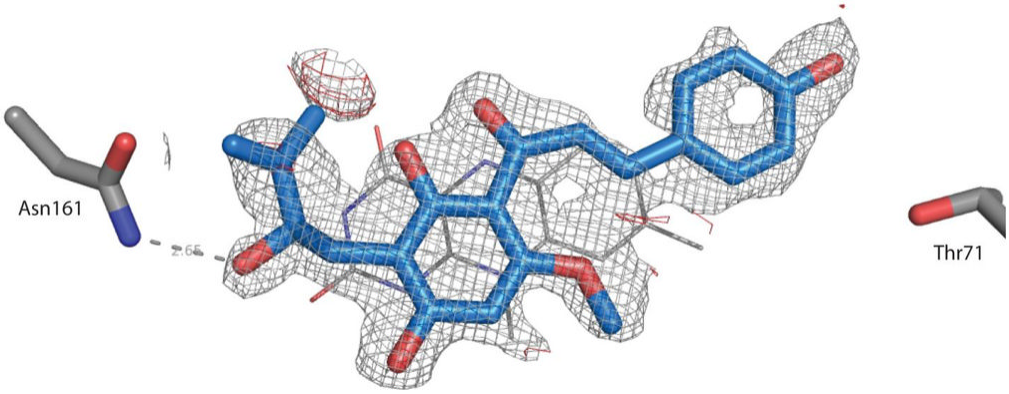

Figure 6.
Crystal structure of QR-2 in complex with xanthohumol D. Xanthohumol D is colored in blue according to atom, and a hydrogen bond is shown as a grey dashed line. The 2Fo-Fc map is shown in grey mesh and contoured to 1.0σ, and the Fo-Fc map is shown in red mesh and contoured to 3.0σ.