NAD(P)H:Quinone Oxidoreductase 1 and NRH:Quinone Oxidoreductase 2 Activity and Expression in Bladder and Ovarian Cancer and Lower NRH:Quinone Oxidoreductase 2 Activity Associated with an NQO2 Exon 3 Single-Nucleotide Polymorphism

David Jamieson, Kerrie Wilson, Simon Pridgeon, Jane P. Margetts, Richard J. Edmondson, Hing Y. Leung, Richard Knox, and Alan V. Boddy

Purpose: NRH:quinone oxidoreductase 2 (NQO2) is a homologue of NAD(P)H:quinone oxidoreductase 1 (NQO1). Despite 54% homology with human NQO1, NQO2 has little endogenous enzymatic activity. However, NQO2 has potential as a therapeutic target because the addition of the nonbiogenic electron donor dihydronicotinamide riboside (NRH) selectively potentiates the bioactivation of the alkylating agent tretazicar (CB1954). The NQO activity of ovarian and bladder tumors was determined and the effect of NQO polymorphisms on NQO activity was investigated.

Experimental Design: Intraperitoneal ovarian metastases and bladder tumor clinical samples were analyzed for NQO1 and NQO2 activity, mRNA expression by semiquantitative reverse transcription-PCR, and genotype by RFLP analysis.

Results: NQO1 activity was higher in the bladder cohort than in the ovarian cohort (0-283 and 0-30 nmol/min/mg, respectively; P < 0.0001). In contrast, NQO2 activity was higher in the ovarian tissue than in the bladder samples (0.15-2.27 and 0.114 nmol/min/mg, respectively; P = 0.0004). In both cohorts, the NQO1 C609T single-nucleotide polymorphism (SNP) was associated with ~7-fold lower NQO1 activity. The NQO2 exon 3 T14055C SNP was associated with lower NQO2 activity relative to wild-type [median values of 0.18 and 0.37 nmol/min/mg in the bladder samples (P = 0.007) and 0.82 and 1.16 nmol/min/mg in the ovarian cohort (P = 0.034)].

Conclusion: This is the first observation reporting an apparent association between an NQO2 exon 3 SNP and lower enzymatic activity. The high NQO2 activity of intraperitoneal ovarian metastases relative to other tissues indicates a potential for tretazicar therapy in the treatment of this disease. In contrast, the low level of NQO1 activity and expression relative to other tissues suggests that NQO1-directed therapies would not be appropriate.

NAD(P)H:quinone oxidoreductase 1 (NQO1, EC 1.6.5.2) is a quinone reductase implicated in protection against carcinogenesis and exploited in cancer chemotherapy (1). NRH:quinone oxidoreductase 2 (NQO2, EC 1.10.99.2) is a homologue of the more fully characterized NQO1 (2). Whereas the two proteins retain similar substrate specificities, structural differences result in differences in affinity for electron donating cofactors. NQO1 can use either NADH or NADPH. In contrast, NQO2 can use neither NADH nor NADPH efficiently and instead requires the nonbiogenic compound dihydronicotinamide riboside (NRH) or related molecules to reveal what can be considered a latent enzymatic activity (3). Both proteins have also been implicated in stabilization of p53 (4, 5) and, in the case of NQO1, this has been shown to result from a direct protein:protein interaction (6).

Polymorphisms have been described for both NQO1 and NQO2. A well-characterized single-nucleotide polymorphism (SNP) in the NQO1 gene (C609T) results in a proline to serine substitution and ubiquitination with rapid degradation of the variant protein (7). Numerous polymorphisms have been reported in NQO2, mostly in the promoter region (8, 9). Increased NQO2 mRNA expression (8), decreased NQO2 expression (10), increased incidence of Parkinson’s disease (8), schizophrenia (11), and alcohol withdrawal symptoms (11) have all been associated with the insertion allele of a 29-bp insertion/deletion promoter polymorphism.

The enzymatic function of NQO1 is a candidate activity in the bioreductive activation of mitomycin C (MMC; ref. 12). MMC is a substrate for NQO1 (12) and MMC toxicity correlates with NQO1 activity in the NCI-60 cell line panel (13). A loss-of-function SNP (C609T) with a genotypic...
frequency of between 4% and 30% has been shown to result in a decreased efficacy of i.p. administered MMC (14). However, MMC is both a poor substrate for and a mechanistic inhibitor of NQO1 (12), and other studies have failed to find an association with NQO1 genotype or expression and MMC efficacy in bladder cancer (15). Two experimental drugs in clinical trial (EO9 and RH1) were specifically designed as NQO1 substrates in the hope that better substrates than MMC would be more selectively toxic to tumors with high levels of NQO1 expression (16, 17).

Tretazicar [5-aziridin-1-yl]-2,4-dinitrobenzamide; formerly designated CB 1954] was developed as an antitumor compound nearly 40 years ago and was characterized as being highly toxic to rat tumors but ineffective in humans (18). This discrepancy is attributable to sequence differences between human and rat NQO1, the rat enzyme bioreductively activating the compound that is not a substrate for human NQO1 (18). Tretazicar is a substrate for human NQO2 when enzymatic activity is facilitated by NRH (3) and dual administration of tretazicar and an NRH analogue (caricotamide) is undergoing phase I trials in the United Kingdom. Initial clinical results are consistent with the very efficient catalytic conversion of tretazicar by NQO2 in the presence of caricotamide.3

A potential problem in the use of enzyme directed bioreductive agents is toxicity in organs expressing high levels of the exploited enzyme, kidney in the case of NQO1 directed therapy (19) and liver in the case of NQO2 (20). This concern can be circumvented if the drug is administered locally rather than systemically. Both superficial bladder cancer and intraperitoneal ovarian metastases are suitable candidates for locally administered chemotherapy. Bladder tumors have previously been reported to express more NQO1 than normal bladder (21). Intravesical administration of MMC is routinely used in the management of superficial bladder cancer and intravesical administration of E09 is being investigated in the same disease (22). I.p. administration of cisplatin and paclitaxel as a treatment for intraperitoneal ovarian metastases may result in an improved overall survival when compared with i.v. administration (23). Phase I data already exist for i.p. administration of tretazicar as a single agent. At an i.v. dose of 24 mg/m², the serum area under the curve was 5.8 μmol/L/h; i.p. administration (24 mg/m²) achieved a peritoneal area under the curve of 387 μmol/L/h, giving a considerable regional advantage (20). Whereas the NQO1 expression of bladder tumors has been investigated, the NQO2 activity of bladder tumors has been ignored and the NQO2 activity of bladder tumors has been investigated, the NQO2 activity of bladder tumors has been investigated, the (21). Intravesical administration of MMC is routinely used in the management of superficial bladder cancer and intravesical administration of E09 is being investigated in the same disease (22). I.p. administration of cisplatin and paclitaxel as a treatment for intraperitoneal ovarian metastases may result in an improved overall survival when compared with i.v. administration (23). Phase I data already exist for i.p. administration of tretazicar as a single agent. At an i.v. dose of 24 mg/m², the serum area under the curve was 5.8 μmol/L/h; i.p. administration (24 mg/m²) achieved a peritoneal area under the curve of 387 μmol/L/h, giving a considerable regional advantage (20). Whereas the NQO1 expression of bladder tumors has been investigated, the NQO2 activity of bladder tumors is unknown. I.p. ovarian metastases have not been characterized for NQO1 or NQO2 activity.

This article reports the expression and activity of NQO1 and NQO2 in cohorts of ovarian metastases and bladder tumors. NQO1 and NQO2 polymorphism frequencies were determined and the effects of the polymorphisms on NQO1 and NQO2 activity and expression were described in both cohorts.

### Materials and Methods

Tretazicar and NRH were supplied by Protherics Plc (Salisbury, United Kingdom). Reagents for PCR and semiquantitative reverse transcription-PCR including Gene Expression Assays primer/probe kits [NQO1, Hs00168547; NQO2, Hs00168552; heat shock 90-kDa protein 1, β (HSPCB), Hs00607336] were from Applied Biosystems (Cheshire, United Kingdom). Agarose and PCR markers were from Promega (Southampton, United Kingdom). Restriction enzymes were purchased from New England Biolabs (Hitchin, United Kingdom). RNaseasy mini kit, DNaseasy Tissue kit, and RNase-Free DNase Set were from Qiagen (Crawley, United Kingdom).

Other chemicals were purchased from Sigma-Aldrich (Poole, Dorset, United Kingdom).

**Protein extraction.** Up to 250 mg of frozen tissue were lysed in 1-mL ice cold lysis buffer [1% (v/v) NP40, 1 μg/mL aprotinin in PBS] and homogenized by an Ultra Turrax tissue homogenizer with two 15-s bursts at 13,500 rpm. The homogenate was transferred to a 1.5-mL Eppendorf tube and centrifuged for 5 min at 4°C and 10,000 × g. The clear supernatant was transferred to a clean Eppendorf tube; 50 μL were removed for protein determination and the remainder stored at −80°C before enzyme analysis. Protein concentration was determined by the bicinchoninic acid assay method.

**DNA and RNA extraction.** DNA and RNA were extracted with Qiagen DNaseasy kit and RNaseasy kit, respectively, according to the manufacturer’s instruction for isolation from animal tissue.

**NQO1 activity assay.** The NQO1 activity of cell lysis supernatants was determined by an adaptation of a previously published method (24). The reaction monitored the reduction of 2,6-dichloroindophenol with and without dicumarol, and the NQO1 activity was taken as the dicumarol-sensitive fraction. Between 1 and 20 μg of total protein were added to a solution containing final concentrations of 0.04 mmol/L 2,6-dichloroindophenol ± 0.01 mmol/L dicumarol in 25 mmol/L Tris-HCl buffer (pH 7.4). The reaction was initiated by the addition of NADH to a final concentration of 0.2 mmol/L and a final volume of 300 μL. The absorbance at 600 nm was measured at 10-s intervals over 2.5 min at room temperature, monitored using a Spectramax 250 spectrophotometer. All reactions were carried out in 96-well plates and each sample was measured in triplicate. The concentration of oxidized 2,6-dichloroindophenol remaining at each time point was calculated from a molar extinction coefficient of 21 mL/μmol/cm. The reaction rate in nanomoles of 2,6-dichloroindophenol reduced per minute per milligram of total protein was calculated from a plot of ΔA600 against time.

**NQO2 activity assay.** Tretazicar reduction was assayed as previously described (25) with minor modifications. An aliquot of cell or tissue lysate (200 μL) was added to a borosilicate tube containing NRH and tretazicar in PBS to give final concentrations of 500 μmol/L NRH and 100 μmol/L tretazicar in a volume of 1 mL at a pH of 7.4. Reactions were incubated at 37°C and 90-μL aliquots were removed at 0, 5, 10, 20, 30, 40, 60, and 80 min; added to 90-μL acetonitrile; vortex mixed; and centrifuged at 12,000 × g for 5 min. The tretazicar concentration remaining at each time point was determined by high-performance liquid chromatography analysis and interpolated from a standard curve. Isocratic high-performance liquid chromatography separation of tretazicar was carried out on a Waters Alliance 2695 separation module and 2487 dual wavelength detector with detection at 325 nm. Fifteen microliters of reaction mixture/acetonitrile (50:50) were injected onto a Genesis C18 120A 4 μm, 100 × 4.6 mm high-performance liquid chromatography column with a mobile phase of 80% 0.02 mol/L phosphate (pH 6):20% methanol at an isocratic flow of 1 mL/min. Tretazicar was detected at a wavelength of 325 nm and a retention time of 7 min, and the concentration remaining was interpolated from a standard curve. The rate of tretazicar reduction activity was calculated by the initial velocity per milligram of protein.

**One-electron assays.** NADH:cytochrome b5 and NADPH:cytochrome c reductase activities were assayed by previously published methods (26, 27), adapted for 96-well plates, using cytochrome c and potassium ferricyanide, respectively, as substrates.

**Semiquantitative reverse transcription-PCR.** cDNA was generated to a concentration equivalent to 20 ng/μL RNA in a volume of 20 μL.
Reaction tubes were incubated at 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min in a GeneAmp PCR System 2700 PCR thermal cycler. Once cooled to 4°C, cDNA was stored at −20°C. cDNA was prepared by the same method to a concentration equivalent to 100 ng/ml to generate a standard curve.

**NQO1 and NQO2 expression.** Expression of NQO1 and NQO2 was determined by semiquantitative real-time PCR relative to HPSCB (28) expression. cDNA diluted to 10 ng in a volume of 11.25 μl was added in triplicate for the gene of interest and endogenous control in a 96-well optical reaction plate. To each well was added TaqMan Universal PCR master mix and TaqMan Gene Expression Assays primer/probe kits to a final volume of 25 μl/well. Quantities of both analytes and endogenous control were interpolated from a standard curve of cDNA derived from RT4 bladder cancer cells. The plates were incubated and monitored in a GeneAmp 5700 sequence detection system per manufacturer’s instructions.

**NQO1 genotyping.** NQO1 PCR and RFLP analysis were carried out to determine the NQO1 genotype of the clinical tissues as previously described (29). Cycling variables were 95°C × 10 min, 45 cycles of 95°C × 1 min, 65°C × 1 min, and 72°C × 1 min, followed by a 10-min extension step at 72°C × 10 min. Digestion products were 20 and 219 bp with the C609 allele and 20, 174, and 45 bp with the 609T allele and were visualized under UV following electrophoresis on a 2% agarose gel prestained with ethidium bromide.

**NQO2 genotyping.** PCR of two NQO2 promoter regions and two exons was carried out to identify previously reported polymorphisms. Primers and cycling conditions for the promoter 1, exon 1, and exon 3 sequences have previously been described (30). Primers for promoter 2 (F-5'-GAGTGGCAGCCGACCTAG-3', R-5'-ACCTAAGCAGCCCGGAC-3') were generated using Primer3 software (31) and PCR was carried out with an annealing temperature of 58°C. Five of the six NQO2 polymorphisms were detected by RFLP analysis. The G3395, G3423A, A3777G, A3968C, and T14055C alleles were identified with the restriction enzymes NlaIV, BstNI, PstI, Cac8I, and SmI, respectively. A 29-bp promoter polymorphism was detected according to migration on a 2% agarose gel prestained with ethidium bromide.

### Results

Forty-two clinical samples were acquired from the bladder tumor bank at the Freeman Hospital, Newcastle upon Tyne. Following transurethral resection, samples were frozen in liquid nitrogen and stored at −80°C until analysis. One sample was subsequently identified as an inflamed, but nonmalignant, biopsy.

Thirty-one gynecologic tumor samples were obtained from patients undergoing primary surgery at the Queen Elizabeth Hospital, Gateshead, United Kingdom. Following surgery, samples were snap frozen in liquid N₂ and stored at −80°C until analysis. Of these samples, three were from primary ovarian tumors, two were from mixed Mullerian tumors, two were classified as borderline tumors, one was of uterine origin, and one was of unknown histology. The remaining 22 samples were intraperitoneal metastases from ovarian adenocarcinomas. Approximately 30% of both cohorts were current smokers. All of the patients from whom samples were obtained provided written, informed consent for their tissues to be used for medical research. Appropriate ethics approval was obtained for both parts of the study.

Of the bladder cohort, DNA was extracted from all of the samples received for genotype analysis. RNA was extracted from 37 of the 42 of the samples, but in 5 samples insufficient RNA was extracted. RNA was used to determine NQO1 and NQO2 expression. Cytosolic protein was extracted from 38 of 42 and used to analyze NQO1, NQO2, cytochrome b₅ reductase, and cytochrome c reductase activity.

Of the gynecologic samples, DNA, RNA, and protein were extracted from all of the samples, although the protein recovery from one sample was insufficient to determine NQO2 activity. Reductase activity was determined in all bladder tumor samples and all ovarian intraperitoneal metastases samples from which sufficient protein was extracted. NQO1 activity was higher in the bladder cohort than in the ovarian cohort with median values of 28 (0-283) versus 3 (0-30) nmol/min/mg (P = 0.0004, Mann-Whitney), respectively. NQO2 activity was significantly lower in the bladder cohort than the ovarian cohort, with median values of 0.27 (0-1.14) versus 1.04 (0.15-2.27) nmol/min/mg (P = <0.0001, Mann-Whitney), respectively. There was no significant difference in cytochrome c reductase (median values of 1.6 and 2.2 nmol/min/mg; P = 0.178, Mann-Whitney) or cytochrome b₅ reductase activity (median values of 140 and 228 nmol/min/mg; P = 0.784, Mann-Whitney) between the bladder and ovarian cohorts, respectively. There was no difference in NQO1 or NQO2 activity between current smokers and nonsmokers in either cohort.

The lower NQO1 activity in the ovarian cohort compared with the bladder cohort was reflected in a lower NQO1 expression (median values, 0.095 versus 0.020; P = 0.0002). In contrast, there was no significant difference in NQO2 expression between the ovarian and bladder cohorts (median values, 0.895 versus 0.850; P = 0.636, Mann-Whitney).

Data from 34 of the bladder cohort were used to investigate the relationship between quinone reductase expression and activity. A correlation was observed between NQO1 activity and expression when one outlier (BC019) with the highest activity was excluded (Fig. 1B). Comparing data for the two quinone reductases together, there was no significant correlation between NQO1 and NQO2 activity or expression. However, a correlation was observed between NQO1 activity and expression when one outlier (BC019) with the highest NQO1 expression (1.45) was excluded from the analysis (r² = 0.220, P = 0.0059; Fig. 1A). There was also a significant correlation between NQO2 activity and NQO2 expression (r² = 0.368, P = 0.0001; Fig. 1B). Comparing data for the two quinone reductases together, there was no correlation between NQO1 and NQO2 activity or expression. However, there was a highly significant correlation between NQO1 activity and NQO1 activity (r² = 0.459, P = 0.001) when two outliers (NQO2OV 1 and 13) with high expression and low activity were excluded (Fig. 1C). In contrast, no correlation was observed between NQO2 activity and expression (Fig. 1D).

As with the bladder tumor samples, a statistically significant correlation was observed between NQO1 activity and cytochrome c reductase activity (r² = 0.242, P = 0.020) but not with cytochrome b₅ reductase activity. There was no correlation between NQO2 activity and either NQO1 activity or either of the one-electron reductase activities.

Data from 22 (or 21 if involving NQO2 activity) ovarian intraperitoneal metastases were used to investigate the relationship between quinone reductase expression and activity. There was a highly significant correlation between NQO1 expression and NQO1 activity (r² = 0.459, P = 0.001) when two outliers (NQO2OV 1 and 13) with high expression and low activity were excluded (Fig. 1C). In contrast, no correlation was observed between NQO2 expression and activity (Fig. 1D).
equilibrium, apart from the NQO2 29-bp insertion/deletion polymorphism. NQO1 allelic frequencies are consistent with published frequencies in a northern European population. NQO2 allelic and genotype frequencies differed from previous observations, with the 3711 29-bp insertion/deletion, A3777G, A3968C, and T14055C variant alleles being less frequent and the C3395C and G3423A variant alleles being more frequent than previously reported (Table 1). The A3777G and T14055C SNP were linked in incidence (P < 0.001) in both cohorts.

In both cohorts, the NQO1 609T allele was associated with a lower median NQO1 activity compared with the C609 allele [CC, 36 nmol/min/mg; CT/TT, 5 nmol/min/mg (P = 0.02, Mann-Whitney) in bladder cohort; CC, 7 nmol min mg; CT/TT, 1 nmol/min/mg (P = 0.011, Mann-Whitney) in ovarian cohort; Fig. 2 A and B]. There was no difference in expression at the RNA level associated with the NQO1 609T allele in either cohort.

In bladder tumor samples, three of the NQO2 SNPs were associated with a lower enzyme activity. Two NQO2 promoter SNPs, the 3423G and 3777G alleles, were associated with lower NQO2 activity when compared with wild-type homozygotes (Table 2). No difference in NQO2 expression at the RNA level was observed between the various alleles. Presence of the exon 3 14055C allele was associated with a lower median activity compared with the wild-type homozygotes (Table 2; Fig. 2C). There was no difference observed in NQO2 expression at the RNA level associated with the NQO2 exon 3 T14055C SNP.

Two other promoter polymorphisms (G3395C and 3711 29-bp insertion/deletion) and an exon 1 SNP (A3968C) were not associated with any difference in NQO2 activity or expression.

**Table 1.** Genotype and allelic frequency of NQO1 and NQO2 polymorphisms in bladder and peritoneal tissue samples

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotype frequency (%)</th>
<th>Allelic frequencies</th>
<th>P (Hardy-Weinberg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NQO1 C609T</td>
<td>CC 43 (58.9) CT 26 (35.6) TT 4 (5.5)</td>
<td>C: 0.77, T: 0.23</td>
<td>0.979</td>
</tr>
<tr>
<td>NQO2 C3395G</td>
<td>CC 30 (41.1) CG 33 (15.2) GG 10 (13.7)</td>
<td>C: 0.64, G: 0.36</td>
<td>0.847</td>
</tr>
<tr>
<td>NQO2 G3423A</td>
<td>GG 22 (30.1) GA 39 (53.4) AA 12 (16.4)</td>
<td>G: 0.57, A: 0.43</td>
<td>0.447</td>
</tr>
<tr>
<td>NQO2 3711 29-bp ID</td>
<td>II 55 (76.4) ID 9 (12.5) DD 8 (11.1)</td>
<td>I: 0.83, D: 0.17</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NQO2 A3777G</td>
<td>AA 46 (63.9) AG 20 (27.8) GG 6 (8.3)</td>
<td>A: 0.78, G: 0.22</td>
<td>0.096</td>
</tr>
<tr>
<td>NQO2 A3968C</td>
<td>AA 39 (53.4) AC 31 (42.5) CC 3 (4.1)</td>
<td>A: 0.75, C: 0.25</td>
<td>0.296</td>
</tr>
<tr>
<td>NQO2 T14055C</td>
<td>TT 53 (72.6) TC 17 (23.3) CC 3 (4.1)</td>
<td>T: 0.84, C: 0.16</td>
<td>0.295</td>
</tr>
</tbody>
</table>

NOTE: All samples were bladder tumors apart from BC020, which was a nonmalignant inflamed biopsy specimen, or peritoneal metastatic samples apart from NQ02hOV 15, 25, and 26, which were primary ovarian.

Abbreviation: ID, insertion/deletion.
In the ovarian cohort, the NQO2 promoter 3777G and exon 3 14055C alleles (Table 2; Fig. 2D) were associated with lower NQO2 activity when compared with wild-type homozygotes. No difference in NQO2 expression at the RNA level was observed between the various alleles.

**Discussion**

The activity of both NQO1 and NQO2 can be exploited in cancer chemotherapy with bioreductive drugs. Five-year survival rates in ovarian cancer have remained at 30% for 20 years (32). New treatment options are needed. The NQO2 activity in intraperitoneal ovarian tumors reported here makes this disease a candidate for treatment with the NQO2 substrate tretazicar:caricotamide. Superficial bladder tumors may also be a suitable for tretazicar:caricotamide therapy. Additionally, NQO2 genotype may be predictive of response to tretazicar:EP015R.

The NQO1 activity and phenotype-genotype relationship of bladder tumor patients reported here are consistent with the literature. Whereas MMC is routinely used in the treatment of superficial bladder cancer, the dependence of MMC efficacy on NQO1 activity is uncertain. MMC cytotoxicity is associated with NQO1 activity *in vitro* (24). However, MMC efficacy did not correlate with NQO1 expression as determined by immunohistochemistry in a retrospective study of a superficial bladder cancer cohort (15). Similarly, there was no association between MMC efficacy and the NQO1 C609T genotype in another retrospective study of superficial bladder cancer (33). This is in contrast to a study that identified an increased efficacy of MMC, associated with the C609T allele in patients with optimally debulked intraperitoneal metastases (14). Whereas NQO1 may not be a major determinant of MMC efficacy in the treatment of SBC, the activity of MMC analogues that have been specifically designed as substrates for NQO1 (e.g., EO9 and RH1) is likely to be more dependent on NQO1 genotype and phenotype (34, 35). Intravesical administration of EO9 in the treatment of SBC is being investigated (22). The efficacy of such compounds *in vivo* in NQO1-null phenotype individuals has not been determined but should be investigated as part of the translational research aspect of clinical trials currently under way.

This is the first reported quantification of NQO1 activity in intraperitoneal ovarian metastases, with a median activity of 3 nmol/min/mg. There was no or negligible NQO1 activity...
detectable in 12 of 31 samples. Whereas NQO1 is fairly ubiquitously expressed in human tissues, the activity in these tumors is one of the lowest reported and less than that seen in normal colon, liver, and lung (36). The median value is an order of magnitude less than that observed in bladder tumor samples (median, 28 nmol/min/mg). This is in apparent contrast to NQO1 expression in healthy ovarian tissue and primary ovarian tumors determined by immunohistochemistry (37, 38). The low NQO1 activity in this cohort indicates that advanced-stage ovarian cancer would not be a suitable target for i.p. NQO1-directed therapies.

The association between NQO1 activity and the C609T SNP in both the bladder and ovarian cohorts is consistent with a large body of literature (1). The activity of NQO2 in bladder cancer reported here (median, 0.27 nmol/min/mg) is low and comparable to that seen in human WBC (0.26 nmol/min/mg). However, the data here show that NQO2 is expressed in superficial bladder tumors, and therefore is potentially a target for tretazicar:caricotamide therapy, with tumor selectivity maintained by intravesicular administration.

The level of NQO2 activity in intraperitoneal ovarian metastases is comparatively high and suggests a potential for NQO2-directed i.p. chemotherapy. A 10-fold increase in cisplatin concentration and a 1,000-fold increase in paclitaxel are obtainable in peritoneal fluid with i.p. administration when compared with i.v. administration (39). I.p. administration of cisplatin and paclitaxel in optimally debulked patients with intraperitoneal ovarian metastases results in increased progression-free and overall survival when compared with i.v. administration (23). There are no data on i.p. administration of tretazicar:caricotamide. However, other bioreductive drugs have been administered i.p. This route of administration of MMC seems to offer improved survival in peritoneal carcinomatosis, but with a mortality rate of between 0% and 14% (40). The high level of NQO2 activity in the liver may raise concerns of hepatotoxicity with i.p. therapy.

In both cohorts, an association was seen between an exon 3 SNP and lower NQO2 activity. This is the first observation of a lower NQO2 activity associated with this SNP. The 29-bp insertion/deletion polymorphism has been associated with a number of neurologic diseases in epidemiologic studies (8, 11, 41). The 29-bp insertion has been associated with higher NQO2 mRNA expression in human WBC (8). In contrast, the 29-bp insertion is associated with a lower NQO2 activity in human fibroblast cells (10). The 29-bp insertion/deletion had no effect on NQO2 activity in the bladder and ovarian cohorts investigated here. However, the highly linked T14055C and A3777G SNPs were associated with 2- and 1.4-fold higher activity in both the bladder and ovarian cohorts, respectively. Neither of these SNPs were associated with differences in NQO2 mRNA expression. It is therefore more likely that the T14055C exon 3 SNP, rather than the A3777G promoter SNP, is responsible for the difference in activity. The 2423A SNP was associated with a lower NQO2 activity in the bladder cohort but not in the ovarian cohort. This was concomitant with linkage in incidence with the T14055C SNP in the bladder cohort, a linkage not observed in the ovarian cohort, which is possibly attributable to the small size of the cohort. The T14055C SNP results in a phenylalanine to leucine substitution (F47L) in a region involved in the dimer:dimer interaction. The phenotype of the protein product of the 14055C determined protein remains to be characterized. It is unknown if the protein product is subject to a rapid ubiquitin-mediated degradation in a mechanism analogous to NQO1 C609T (7). Whereas Western blotting would have provided an indication about the mechanism by which NQO2 activity is lower in the 14055C SNP protein, there was insufficient material to undertake both immunoblot and activity assays in this study. We decided that an activity assay would be the appropriate direction to take in this study as any SNP resulting in loss of activity without loss of protein would not have been observed. The specificity of the assay is supported by cell line data wherein V79 cells have no detectable NQO2 activity unless transfected with hRNQO2. NQO2 activity is not detected in the same cell line transfected with NQO1 in the presence of NRH (43).

<table>
<thead>
<tr>
<th>Genotype and NQO2 activity (nmol/min/mg)</th>
<th>P (Mann-Whitney)</th>
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<tbody>
<tr>
<td>NQO2 C3395G</td>
<td></td>
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<tr>
<td>Bladder</td>
<td>0.36</td>
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<tr>
<td>Ovarian</td>
<td>0.545</td>
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<td>NQO2 G3423A</td>
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<td>Bladder</td>
<td>0.17</td>
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<tr>
<td>Ovarian</td>
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<td>NQO2 3711 29-bp ID</td>
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<tr>
<td>Bladder</td>
<td>0.37</td>
</tr>
<tr>
<td>Ovarian</td>
<td>1.16</td>
</tr>
</tbody>
</table>

Knox et al., Cancer Res, submitted.
There is a trend towards lower NQO2 activity associated with an NQO2 exon 3 T14055C SNP in both the bladder and ovarian tissue cohorts compared with wild-type homoyogotes. The cohort sizes used in this study are comparable and ovarian tissue cohorts compared with wild-type homozygotes.

The implication for tretazicarbicamidate of this SNP is uncertain and should be investigated as part of clinical trials. The predictive power of NQO1 genotype on MMC efficacy is unclear and seems to be tumor specific (14, 15). The function of NQO2 in the reductive activation of tretazicarbicamidate is much clearer, and it is likely that tumors homoyozygous for the T14055C SNP will be unresponsive to tretazicarbicamidate.

References
NAD(P)H:Quinone Oxidoreductase 1 and NRH:Quinone Oxidoreductase 2 Activity and Expression in Bladder and Ovarian Cancer and Lower NRH:Quinone Oxidoreductase 2 Activity Associated with an NQO2 Exon 3 Single-Nucleotide Polymorphism

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