Polyhalogenated benzo- and naphthoquinones are potent inhibitors of plant and bacterial ureases

Ainura Ashiralieva, Diethelm Kleiner*

Mikrobiologie, Universität Bayreuth, 95440 Bayreuth, Germany

Received 19 August 2003; revised 22 September 2003; accepted 4 November 2003

First published online 12 November 2003

Edited by Judit Ovádi

Abstract Polyhalogenated benzo- and naphthoquinones were found to be potent inhibitors of pure ureases from *Bacillus pasteurii* and *Canavalia ensiformis*. They also inhibited ureases in whole cells of *Helicobacter pylori*, *Klebsiella oxytoca* and *Proteus mirabilis*. Inhibition was non-competitive with K_i values in the micromolar range or below. Inhibition was irreversible as shown by equilibrium dialysis. Inhibitory power decreased considerably when halogens were replaced by –OH, –CN, alkoxy or alkyl groups.

Key words: Urease; Enzyme inhibition; Quinone; Fluoranil; Chloranil; Bromanil

1. Introduction

Ureases (urea amidohydrolases: E.C. 3.5.1.5) are multi-subunit, nickel-containing enzymes that catalyze the hydrolysis of urea to carbon dioxide and ammonia.

Bacterial ureases have been shown to be important virulence factors in the pathogenesis of many diseases in humans and animals [1,2]. The ammonia generated may severely disturb metabolic functions in a large number of animal tissues and organs [3,4]. Ammonia is involved in the formation of infection stones in the urinary tract, which cause significant health problems and damage [1,2,5]. Urease is also indispensable for colonization of human gastric mucosa by *Helicobacter pylori*. The ammonia produced has been shown to be toxic for various gastric cell lines [2]. Furthermore, urease activity was proposed to damage the gastric epithelium via its interaction with the immune system by stimulating an oxidative burst in human neutrophils [6]. H_2O_2 generated in this oxidative burst probably reacts with ammonia and chloride to yield the toxic monochloramine [7,8]. Finally, the ammonia may reach the serum and contribute to symptoms of hepatic encephalopathy in patients suffering from cirrhosis [9]. On site ammonia itself inhibits lymphokine release [10] and the fourth component of complement [11], thus lowering the cell-mediated immune response and host defence mechanisms. The consequences are acute inflammation and epithelial cell necrosis.

Apart from ammonia, the carbon dioxide generated by urea hydrolysis may play a significant role for survival of *H. pylori* in the gastric mucosa. Kuwahara et al. [12] could show that this CO_2 rapidly neutralizes peroxynitrite ($ONOO^-$), which is produced by the host, and which efficiently kills *H. pylori*. They demonstrated that a raise in local CO_2 concentration significantly enhanced the survival rate of the bacteria.

Therefore, strategies based on inhibition of urease are considered as treatment for infections caused by urease-producing bacteria. The discovery of urease inhibitors has mainly relied upon random screening of thousands of chemical compounds. However, after determination of X-ray structures of native and inhibited ureases a more directed search for inhibitors has started (reviewed in [13]).

So far urease inhibitors can be divided into six major classes based on their chemical structure [13]: hydroxamic acid derivatives, phosphorodiamidates (PPD), imidazole derivatives, phosphazenes, Ecabet sodium and N-substituted hydroxyureas [14]. Several compounds (mainly acet-hydroxamic acid and the PPD derivative flurofamide) have proved to be effective in vivo, reducing, e.g., urethral catheter encrustation by *Proteus mirabilis* [15], *Ureaplasma* infection in animals [16,17] or *Helicobacter*-induced gastritis in Mongolian gerbils [18]. These studies indicate that urease inhibitors have the potential to replace, e.g., the current treatment for peptic ulcer based on the combination of an acid pump inhibitor and two antibiotics [19], which is expensive and prone to development of antibiotic resistances.

We here report inhibition of various ureases by novel classes of potent inhibitors – halogenated benzo- and naphthoquinones.

2. Materials and methods

2.1. Enzymes and chemicals

Ureases from *Bacillus pasteurii* and jack bean (*Canavalia ensiformis*), and chloranilic acid were from Sigma (cat. no. U7127, U4002, C-8136, respectively); tetrafluoro-*p*-benzoquinone (*p*-fluoranil, 10,435-3), tetrachloro-*o*-benzoquinone (*o*-chloranil, T660-1), 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (D6,040-0), tetrachlorocatechol (T690-3) from Aldrich; tetrachloro-*p*-benzoquinone (*p*-chloranil, 802361), tetrabromo-*p*-benzoquinone (*p*-bromanil, 820180), *p*-naphthoquinone (806215) from Merck; 2,3-dichloro-*p*-naphthoquinone (35990) from Fluka; *p*-benzoquinone, *o*-naphthoquinone, 2-methyl-6-methoxy-*p*-benzoquinone were gifts from K. Dettner (Department of Animal Ecology, University of Bayreuth). Dibromo-diiodo-*p*-benzoquinone (probably a mixture of isomers) and tetraiodo-*p*-benzoquinone (iodanil) were prepared and purified as described in [20]. The structural formulas of these compounds are given in Fig. 1, and the corresponding names in Table 1.

*Corresponding author. Fax (49)-921-552727.
E-mail address: diethelm.kleiner@uni-bayreuth.de (D. Kleiner).

0014-5793/03/$22.00 © 2003 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)01289-4

2.2. Bacterial strains and their cultivation

Klebsiella oxytoca M5al (from R.H. Burris, Madison, WI, USA) and *P. mirabilis* DSM30115 were grown in 20 ml minimal medium [21] with 1% glucose and 5 mM urea as sole C and N sources (urea stock solution 1 M, filter sterilized); *H. pylori* strain Hp151 (from T. Jahns, Saarbrücken, Germany) in 100 ml brucella broth pH 7.0 (Difco, Detroit, MI, USA) with 2% fetal calf serum (Serva, Heidelberg, Germany) and 10 mg vancomycin (Sigma Aldrich, Deisenhofen, Germany) under microaerobic conditions (85% N_2, 10% CO_2, 5% O_2). All cultivations were carried out at 37°C.

2.3. Assay conditions

Bacteria pelleted from liquid cultures were washed 3× with 10 mM potassium phosphate buffer (pH 7.0) and used in whole cell assays. Urease activity was routinely determined as ammonia formation [22] in a test mixture containing in a final volume of 0.8 ml: urea (0.25 M, if not indicated otherwise), potassium phosphate (25 mM pH 7.0) and variable amounts (5–50 μl) of urease or bacterial cells, and inhibitors as indicated. Inhibitors were dissolved in *tert*.-butyl-methyl ether (stock solutions of 0.01–10 g l^{-1}, the solvent proved non-inhibitory up to a 10% concentration.), pure ureases in 10 mM potassium phosphate pH 7.0. Protein content was determined by the microbiuret method [23].

2.4. Reversibility tests

Reversibility of binding was tested by equilibrium dialysis. 0.25 ml of urease solution (1 g l^{-1}) was mixed with different amounts of inhibitors, left for 30 min at room temperature, placed into a dialysis tube (Visking, molecular weight cut-off 14 000, diameter 7 mm), and dialyzed for up to 22 h against five changes of 100 ml 10 mM potassium phosphate pH 7.0 (20°C). Activity tests were routinely run at 0, 5 and 22 h.

3. Results

3.1. Inhibitory powers

As shown in Table 1 the K_i values for inhibition of pure ureases decrease about 50-fold with halogenation of benzoquinone. Other substituents did not have the same or even adverse effects. The nature of the halogens seemed of less importance. A calculation for the plant urease showed that at the concentration employed (0.046 μM, molecular weight 273 kDa) [13] inactivation took place at an almost stoichiometric 2:1 ratio of some anils to urease, indicating a specific site-directed reaction. Inhibition always proved to be non-competitive. For a rough calculation of the K_i values the formula for pure non-competitiveness ($K_i = [I]/(V_{max}/v - 1)$, $[I]$ = inhibitor concentration) was considered sufficient.

Naphthoquinones were less effective. Tetrachlorocatechol had no effect.

When testing whole cells, passage of the inhibitors across the membrane had to be taken into account. Generally cells and inhibitors had to pre-incubated to obtain stable values. Pre-run experiments showed an incubation time of 2 h for all inhibitors and cells to be sufficient. (Table 2.)

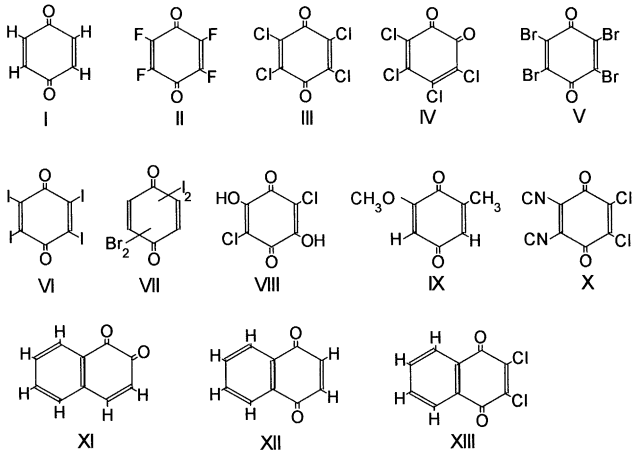

Fig. 1. Structural formulas for the quinones used in this study.

3.2. Reversibility of binding

Reversibility of binding is conveniently studied by equilibrium dialysis. Since successful dialysis depends on solubility of the inhibitor in the dialysis buffer, relevant data had to be collected or produced. A literature search yielded no data for fluoranil or the naphthoquinones, and 'insoluble in water' for chloranil, bromanil and iodanil [20,24,25]. Our own experiments (data not shown) showed a solubility of approximately 0.5 mM for fluoranil and 0.05 mM for chloranil in the dialysis buffer. Both values by far exceed the solubility required for dialysis experiments by more than 10-fold. Fig. 2 shows the equilibrium dialysis data for fluoranil. As can be seen, inhibition of the urease is not relieved even after 22 h of dialysis. Similar data were obtained for chloranil (not shown). These data strongly suggest irreversible binding of halogenated benzoquinones to ureases.

4. Discussion

The results presented here demonstrate that halogenated quinones are potent, irreversible inhibitors of several bacterial and plant ureases. Other substituents tested (–OH, –CN, $–OCH_3$, $–CH_3$) did not increase or rather decreased the inhib-

Table 1
Inhibition constants (K_i in mM) of quinones for pure ureases. Roman numerals in the brackets correspond to the structural formulas in Fig. 1

Inhibitor	*B. pasteurii* urease	*C. ensiformis* urease
p-Benzoquinone (I)	80	10
p-Fluoranil (II)	0.1	0.1
p-Chloranil (III)	0.2	0.2
o-Chloranil (IV)	4.8	3.5
p-Bromanil (V)	0.15	0.3
p-Iodanil (VI)	1.0	0.1
Di-Br,di-I-benzoquinone (VII)	0.2	0.1
Chloranilic acid (VIII)	>1000	100
2-CH_3,-6-OCH_3-benzoquinone (IX)	200	120
2,3-Cl,-5,6-CN-benzoquinone (X)	600	70
o-Naphthoquinone (XI)	70	5
p-Naphthoquinone (XII)	10	7
2,3-Di-Cl-naphthoquinone (XIII)	13	11

Table 2
IC_{50} values (50% inhibition, in μM) for inhibition of ureases in whole bacterial cells. Roman numerals in the brackets correspond to the structural formulas in Fig. 1

Inhibitor	*K. oxytoca*	*H. pylori*	*P. mirabilis*
p-Benzoquinone (I)	–	–	18
p-Fluoranil (II)	25	2.5	7.2
p-Chloranil (III)	13	1	3.7
p-Bromanil (V)	8	2.9	188
p-Iodanil (VI)	>300	3	235
Di-Br,di-I-benzoquinone (VII)	67	1	38
o-Naphthoquinone (XI)	–	–	63
p-Naphthoquinone (XII)	–	–	5
2,3-Di-Cl-naphthoquinone (XIII)	–	–	150

itory power of the unsubstituted quinones. The mechanism of inhibition remains unknown. As far as we know enzyme inhibition by these compounds has only been found for bacterial collagenases which are strongly and irreversibly inactivated by *o*-chloranil but rather weakly by *p*-chloranil. This inactivation was found to be due to reaction with a tyrosyl residue at the active site [26].

Irreversibility and strong inhibition are important prerequisites for potential application as drugs against ureolytic bacteria. Many quinones, however, have been shown to be cytotoxic. Two general mechanisms have been elucidated: oxidative stress following generation of reactive oxygen species due to enzymatic redox cycling and loss of protein thiols due to sulfhydryl arylation [27,28]. But the tyrosine modification mentioned above shows that more possibilities of interaction seem to exist.

Unsubstituted *p*-benzoquinone is highly toxic with an LD_{50} value (lethal dose 50% kill) of 25–100 mg kg^{-1} for mice (http://www.cdc.gov/niosh/rtecs/dk280de8.html), *p*-naphthoquinone a little less (140–400 mg kg^{-1}; http://ntp-support.niehs.nih.gov/NTP_Reports/NTP_Chem_HS_HTML/NTP_Chem1/Radian130-15-4.html). Values for halogenated compounds are scarce, but suggest a significantly reduced toxicity. For *p*-chloranil an LD_{50} of 4 g kg^{-1} rat is reported (http://www.cdc.gov/niosh/rtecs/dk682428.html). Lower concentrations, however, might be cancerogenic. In any case, none of the halogenated compounds used in this study is marked 'toxic' by the manufacturer as are benzo- and naphthoquinone. Most of them are classified as 'irritant'.

Acknowledgements: We thank the DAAD for a grant to A.A., T. Jahms (Saarbrücken, Germnay) for *Helicobacter* samples and K. Dettner (Bayreuth, Germany) for specific quinones.

References

[1] Mobley, H.L.T. and Hausinger, R.P. (1989) Microbiol. Rev. 53, 85–108.
[2] Mobley, H.L.T., Island, M.D. and Hausinger, R.P. (1995) Microbiol. Rev. 59, 451–480.
[3] Visek, W.J. (1982) J. Dairy Sci. 67, 481–498.

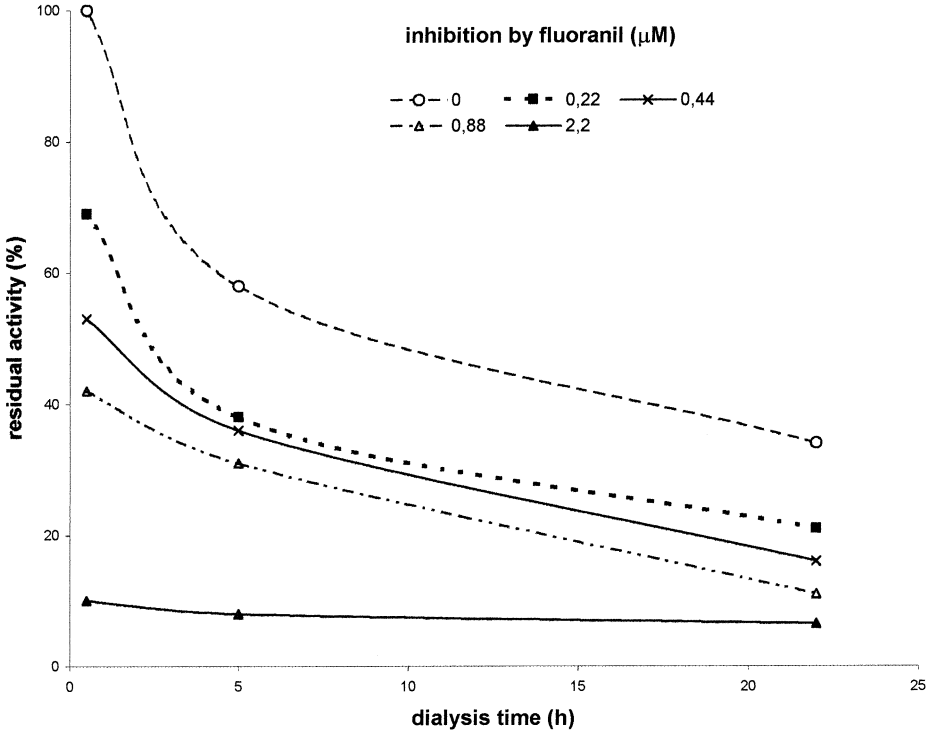

Fig. 2. Dialysis of *C. ensiformis* urease after inhibition by various concentrations of fluoranil: ○ 0; ■ 0.22; × 0.44; △ 0.88; ▲ 2.2 mM.

[4] Kleiner, D., Traglauer, A. and Domm, S. (1998) Bull. Inst. Pasteur 96, 257–265.
[5] McLean, R.J.C., Nickel, J.C., Cheng, K.J. and Costerton, J.W. (1988) CRC Crit. Rev. Microbiol. 16, 37–79.
[6] Suzuki, M., Miura, S., Suematsu, M., Fukumura, D., Kurose, I., Suzuki, H., Kai, A., Kudoh, Y., Ohashi, M. and Tsuchiya, M. (1992) Am. J. Physiol. 263, G719–G725.
[7] Grisham, M.B., Jefferson, M.M. and Thomas, E.L. (1984) J. Biol. Chem. 259, 6766–6772.
[8] Thomas, E.L., Grisham, M.B. and Jefferson, M.M. (1983) J. Clin. Invest. 72, 441–454.
[9] Miyaji, H., Azuma, T., Ito, S., Suto, H., Ito, Y., Yamazaki, Y., Sato, F., Hirai, M., Kuriyama, M., Kato, T. and Kohli, Y. (1997) Aliment. Pharmacol. Ther. 11, 1131–1136.
[10] Targowsky, S.P., Klucinski, W., Babiker, S. and Nonnecke, B.J. (1984) Infect. Immun. 43, 289–293.
[11] Beeson, P.B. and Rowley, D.R. (1959) J. Exp. Med. 110, 685–697.
[12] Kuwahara, H., Miyamoto, Y., Akaike, T., Kubota, T., Sawa, T., Okamoto, S. and Maeda, H. (2000) Infect. Immun. 68, 4378–4383.
[13] Amtul, Z., Rahman, A.U., Siddiqui, R.A. and Choudhary, M.I. (2002) Curr. Med. Chem. 9, 1323–1348.
[14] Uesato, S., Hashimoto, Y., Nishino, M., Nagaoka, Y. and Kuwajima, H. (2002) Chem. Pharm. Bull. (Tokyo) 50, 1280–1282.
[15] Morris, N.S. and Stickler, D.J. (1998) Urol. Res. 26, 275–279.
[16] Furr, P.M., Taylor-Robinson, D. and Hetherington, C.M. (1984) J. Antimicrob. Chemother. 14, 613–618.
[17] Ligon, J.V. and Kenny, G.E. (1991) Infect. Immun. 59, 1170–1171.
[18] Ohta, T., Shibata, H., Kawamori, T., Iimuro, M., Sugimura, T. and Wakabayashi, K. (2001) Biochem. Biophys. Res. Commun. 285, 728–733.
[19] Vaira, D., Holton, J., Ricci, C., Basset, C., Gatta, L., Perna, F., Tampieri, A. and Miglioli, M. (2002) Aliment. Pharmacol. Ther. 16 (Suppl. 4), 105–113.
[20] Torrey, H.A. and Hunter, W. (1912) J. Am. Chem. Soc. 34, 702–716.
[21] Pengra, R.M. and Wilson, P.W. (1958) J. Bacteriol. 75, 21–25.
[22] Fawcett, J.K. and Scott, J. (1960) J. Clin. Pathol. 13, 156–161.
[23] Goa, J. (1953) Scand. J. Clin. Lab. Invest. 5, 218–222.
[24] Beilstein (1925) Handbuch der organischen Chemie, Vol. 7, p. 637, Julius Springer, Berlin.
[25] Beilstein (1925) Handbuch der organischen Chemie, Vol. 7, p. 643, Julius Springer, Berlin.
[26] Makinen, P.-L. and Makinen, K.K. (1988) Biochem. Biophys. Res. Commun. 153, 74–80.
[27] Cadenas, E. (1989) Annu. Rev. Biochem. 58, 79–110.
[28] Bolton, J.L., Trush, M.A., Penning, T.M., Dryhurst, G. and Monks, T.J. (2000) Chem. Res. Toxicol. 13, 135–160.