uptake of anticoagulants such as dicoumarol and warfarin is prevented by their complexation with albumin,[26] and a higher concentration of dicoumarol (200 to 400 μM) must be used. Thus, the effective concentration of inhibitor acting on cells is difficult to control. It is expected that the use of irreversible inhibitors of NQO1 activity, such as the newly developed ES936,[12] and genetic methods that induce a decrease in NQO1 gene expression will help to elucidate the particular role played by NQO1 in the control of cell growth and death.

Acknowledgments

Supported by grants BMC2002–01078 and BMC2002–01602 (Spanish Ministerio de Educación y Cultura and Ministerio de Ciencia y Tecnología) and CVI-276 (Junta de Andalucía). R. I. Bello acknowledges financial support by the Spanish Ministerio de Educación y Cultura. C. Gómez-Díaz was supported by CVI-276.

[26] W. D. Wosilait, M. P. Ryan, and K. H. Byington, *Drug Metab. Dispos.* **9,** 80 (1981).

[14] The "Prochaska" Microtiter Plate Bioassay for Inducers of NQO1

By JED W. FAHEY, ALBENA T. DINKOVA-KOSTOVA, KATHERINE K. STEPHENSON, and PAUL TALALAY

Introduction

The microtiter plate bioassay for NQO1 was first developed in this laboratory by Hans Prochaska as a rapid and direct assay of quinone reductase (NQO1; QR; DT-diaphorase) activity in cultured cells, suitable for identifying, purifying and determining the potency of inducers of this detoxication enzyme.[1,2] De Long *et al.*[3] were the first to suggest exploiting the specific activities of NQO1 in Hepa 1c1c7 murine hepatoma cells for bioassay of the inducer potencies of phase 2 enzymes. These cells mimic animal tissues in responding to a wide variety of chemoprotective agents.[3] Hepa 1c1c7 cells were originally established from a transplantable murine

[1] H. J. Prochaska and A. B. Santamaria, *Anal. Biochem.* **169,** 328 (1988).
[2] H. J. Prochaska, A. B. Santamaria, and P. Talalay, *Proc. Natl. Acad. Sci. USA* **89,** 2394 (1992).
[3] M. J. De Long, H. J. Prochaska, and P. Talalay, *Proc. Natl. Acad. Sci. USA* **83,** 787 (1986).

0076-6879/04 $35.00

hepatoma of the C57L/J mouse and the 1c1c7 clone was selected for its highly inducible aryl hydrocarbon hydroxylase (AHH), making this line important in the analysis of the functional characteristics of the *Ah* (*A*ryl *h*ydrocarbon) receptor.[4] The Hepa 1c1c7 line was chosen for the NQO1 assay because: (a) these cells have many characteristics of normal tissues, in particular the capacity for carcinogen activation and xenobiotic metabolism; (b) these cells are amenable to strict control of environmental, nutritional, and hormonal factors; (c) mutants defective in *Ah* receptor or its gene product are available – this is not true for all cells in culture; and (d) these cells have a highly inducible AHH as well as other inducible cytochromes P450, cytochrome P450 reductase, and epoxide hydrolase, thus facilitating metabolic activation of xenobiotics.[3,5]

As adapted for use in microtiter plates, the cells were grown for 24 h, followed by exposure to inducing agents for an additional 24–48 h, and then lysed with digitonin. Cell lysates were assayed for NQO1 activity using a reaction mixture in which both the NADPH and the quinone are regenerated, thus avoiding reagent depletion (Fig. 1). Cell density, as a proxy for total cellular protein, was assayed in replicate plates by vital staining. This assay has proven to be exceptionally useful for its originally envisioned purpose, as well as for the characterization of phase 2 enzyme inducers in a variety of materials. Although other microtiter plate assays have been proposed,[6] none are as responsive or as versatile as the Prochaska assay. The dynamic response of the assay is very wide, permitting the evaluation of compounds over a concentration range of approximately 5 orders of magnitude (Fig. 2).[7]

We have made certain modifications to the assay in order to make it more efficient, and considerable data are now available from laboratories worldwide that support its utility in cancer chemoprevention studies. For example, we have accumulated more than 10 years of baseline data with a single standard NQO1 inducer, β-naphthoflavone (BNF), that has been used in hundreds of separate assays. We have also made observations on the use of this basic assay: (a) with other cell lines and passage numbers, (b) with various common solvent systems, (c) with a wide array of samples, (d) with multiple researchers, (e) as a versatile tool for screening crude plant extracts, (f) as a powerful aid in the directed synthesis of biologically active compounds, and (g) as a dynamic system to monitor conversion of

[4] J. P. Whitlock, *Ann. Rev. Pharm. Toxicol.* **39,** 103 (1999).
[5] M. J. De Long, A. B. Santamaria, and P. Talalay, *Carcinogenesis* **8,** 1549 (1987).
[6] M. Zhu and W. E. Fahl, *Anal. Biochem.* **287,** 210 (2000).
[7] T. Prestera, Y. Zhang, S. R. Spencer, C. A. Wilczak, and P. Talalay, *Adv. Enzyme Regul.* **33,** 281 (1993).

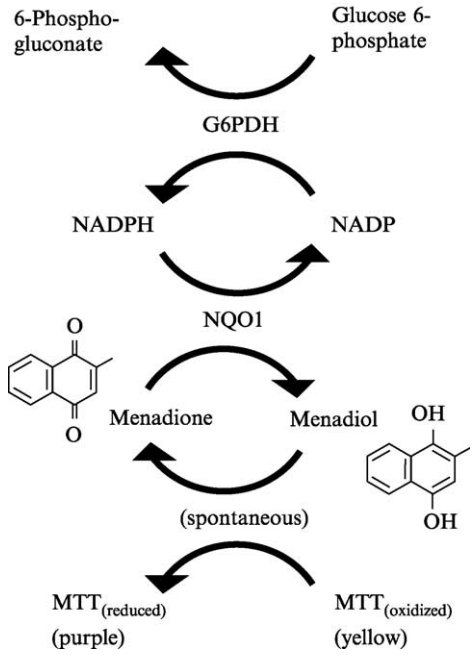

FIG. 1. Principle of the NQO1 assay: Glucose 6-phosphate and glucose-6-phosphate dehydrogenase (G6PDH) generate NADPH continually. This NADPH is used by NQO1 to transfer electrons to menadione. The menadiol thus formed reduces MTT (yellow when oxidized), spontaneously, to the purple formazan which can be measured over a broad range of wavelengths (490 to 640 nm). Both NADPH and menadione are regenerated, which obviates problems encountered with substrate depletion.

inactive precursors to active products. The use of this assay has led to the discovery of a number of potent chemoprotective agents from plants, to their use in controlled animal and human clinical studies, and to the development of similarly active compounds by synthetic means. These findings are reviewed and discussed herein.

Prochaska Bioassay Protocol

As initially described, the induction of NQO1 was measured in Hepa 1c1c7 murine hepatoma cells grown in 96-well microtiter plates containing α-MEM culture medium supplemented with 10% fetal calf serum.[1,2] Approximately 10,000 cells were plated into flat-bottomed, untreated plastic microtiter plates and incubated for 24 h at 37° in a 5% CO_2 atmosphere,

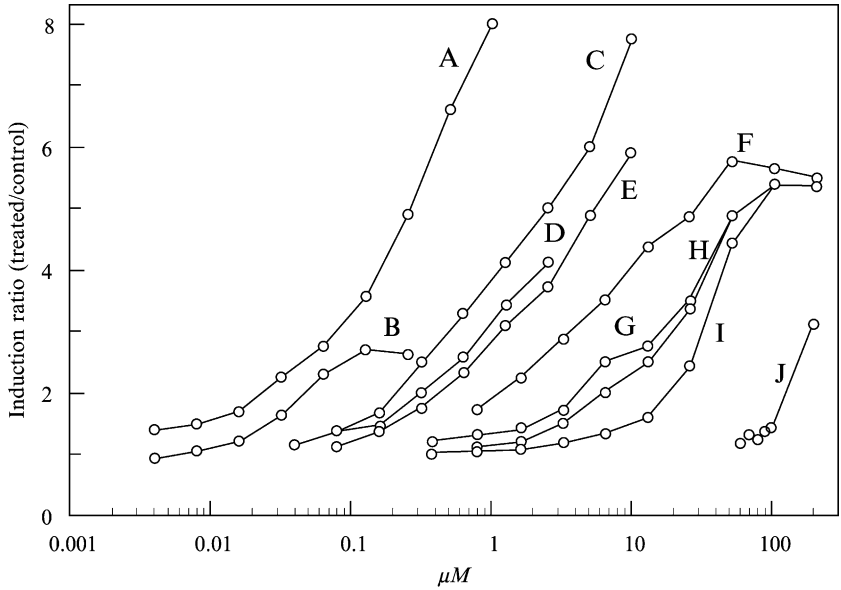

FIG. 2. Dynamic range of the Prochaska Assay. Induction profiles in Hepa 1c1c7 cells are shown for (A) BNF, (B) phenylarsine oxide, (C) sulforaphane, (D) mercury chloride, (E) 1-nitro-1-cyclohexane, (F) 1,3-dithiole-3-thione, (G) benzyl isothiocyanate, (H) 1-butylhydroquinone, (I) BAL, and (J) 1-butylhydroperoxide which had CD's of about 0.02, 0.05, 0.2, 0.3, 0.4, 1.5, 4, 5, 20, and 110 μM, respectively.

during which time they became adherent. Culture medium was then replaced with 150 μL of fresh medium containing antibiotics in order to permit the introduction of non-sterile test compounds (e.g., crude plant extracts). Cells were exposed to inducers for an additional 24 h. Typically the solution to be assayed in DMSO was diluted with cell culture medium and 2-fold serial dilutions were introduced into 8-well columns on the microtiter plates with a multichannel pipettor. The final concentration of DMSO was 0.1% by volume. The plates were then incubated for 24 h, and the medium was discarded. Cells were then lysed with digitonin in the presence of EDTA for 20 min and NQO1 was assayed by addition of an NADPH-generating system that maintained a constant NADPH concentration (glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP), FAD, menadione (2-methyl-1,4-naphthoquinone, a quinone that is reduced to menadiol by NQO1 in the presence of NADPH), and MTT (3-[4,5-dimethylthiazo-2-yl]-2,5-diphenyltetrazolium bromide; a tetrazolium dye that is reduced non-enzymatically by menadiol). NQO1 activity

was stopped after 5 min by the addition of dicumarol (a specific and very potent inhibitor of NQO1), and the reduced formazan dye was measured spectrophotometrically in a 96-well plate reader at 610 nm. Since all wells have negligible absorbance at zero time, the absolute absorbance at 5 min accurately reflects the change in absorbance during the incubation period. Cytotoxicity and cell density were assessed by crystal violet staining of a duplicate set of plates, followed by scanning at 610 nm[1] or 490 nm.[2] One unit of inducer activity is defined as the *Concentration* that *Doubles* the NQO1 specific activity in a microtiter well containing 150 μL of medium. This concentration has been designated the "CD" value. Hence a compound with a CD of 1.0 μM has a potency of 6667 units of inducer activity per μmol.

Technical Improvements of the Assay

The original procedure has been modified in several ways:

1. The fetal calf serum is treated with activated charcoal (1 g/100 ml) for 90 min at 55°,[8] thereby lowering the basal NQO1 levels.

2. Protein content is measured in an aliquot of cell lysates, thereby providing a more rapid and reliable assessment of cell density and eliminating the need for a duplicate set of plates. Protein concentration is measured in a 20-μL aliquot of the digitonin cell lysate in a separate 96-well microtiter plate. Bicinchoninic acid reagent (300 μL)[9] is added and the product is measured spectrophotometrically (550 nm) after 30 min incubation at 37.5°.[10]

3. The exposure period has been increased from 24 to 48 h which magnifies the amplitude of NQO1 induction for many inducers in this cell line.[2,8,10]

4. An automated microtiter plate washer has been used to remove growth medium and rinse cells just before conducting the assay, and the MTT concentration is monitored at 490 nm.[10]

5. We have introduced the use of various solvents in addition to DMSO. Acetonitrile,[2] and a "triple solvent" composed of a mixture of equal volumes of acetonitrile, DMSO, and dimethyl formamide,[10] are both extremely effective solvents for extraction of plant tissues, and they can be diluted into cell culture medium to final concentrations of 1% and 0.5%,

[8] S. R. Spencer, C. A. Wilczak, and P. Talalay, *Cancer Res.* **50,** 7871 (1990).
[9] P. K. Smith, R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk, *Anal. Biochem.* **150,** 76 (1985).
[10] J. W. Fahey, Y. Zhang, and P. Talalay, *Proc. Natl. Acad. Sci. USA* **94,** 10367 (1997).

respectively, without affecting cell viability. Other solvents such as tetrahydrofuran and methanol[11] can be used for more lipophilic compounds, but these solvents must be used at less than 0.1% and 0.33%, respectively, in order to avoid cytotoxicity.

6. We have typically expressed the inducer potency of plant extracts in units/g fr. wt. (fresh weight) or dry wt. Cytotoxicity is of concern if it occurs at or near the concentration range that results in NQO1 induction. Pezzuto and colleagues[12,13] have proposed using a *Chemoprotective Index* (CI; CI = IC_{50}/CD) which provides a useful metric of the window between activity and toxicity.

7. The assay has been adapted by plant scientists[14–16] for rapid screening of individual plants, by introducing leaf disc punches as sources of NQO1 inducer (glucosinolates/isothiocyanates), placed in individual wells of microtiter plates and then removed prior to assay.

Novel Findings Made with The Prochaska Bioassay

The Prochaska microtiter plate bioassay has been exceptionally useful for identifying and isolating inducers of NQO1 from natural sources, and in guiding the synthesis of more potent analogs of isothiocyanates,[12,17,18] dithiolethiones,[19–21] and curcuminoids.[22,23] It has also been used to survey,

[11] F. Khachik, J. S. Bertram, M.-T. Huang, J. W. Fahey, and P. Talalay, *in* "Proceedings of the International Symposium on Antioxidant Food Supplements in Human Health" (L. Packer, M. Hiramatsu, and T. Yoshikawa, eds.), pp. 203–229. Academic Press, New York, 1999.

[12] C. Gerhäuser, M. You, J. Liu, R. M. Moriarity, M. Hawthorne, R. G. Mehta, R. C. Moon, and J. M. Pezzuto, *Cancer Res.* **57,** 272 (1997).

[13] C. Gerhäuser, K. Klimo, E. Heiss, I. Neumann, A. Gamal-Eldeen, J. Knauft, G. Y. Liu, S. Sitthimonchai, and N. Frank, *Mutat. Res.* **523–524,** 163 (2003).

[14] H. B. Gross, T. Dalebout, C. D. Grubb, and S. Abel, *Plant Sci.* **159,** 265 (2000).

[15] Q. Wang, C. D. Grubb, and S. Abel, *Phytochem. Anal.* **13,** 152 (2002).

[16] C. D. Grubb, H. B. Gross, D. L. Chen, and S. Abel, *Plant Sci.* **162,** 143 (2002).

[17] G. H. Posner, C. G. Cho, J. V. Green, Y. Zhang, and P. Talalay, *J. Med. Chem.* **37,** 170 (1994).

[18] Y. Zhang, T. W. Kensler, C. G. Cho, G. H. Posner, and P. Talalay, *Proc. Natl. Acad. Sci. USA* **91,** 3147 (1994).

[19] P. A. Egner, T. W. Kensler, T. Prestera, P. Talalay, A. H. Libby, H. H. Joyner, and T. J. Curphey, *Carcinogenesis* **15,** 177 (1994).

[20] T. W. Kensler, *Environ. Health Perspect.* **4**(Suppl. 105), 965 (1997).

[21] T. W. Kensler, T. J. Curphey, Y. Maxiutenko, and B. D. Roebuck, *Drug Metabol. Drug Interact.* **17,** 3 (2000).

[22] A. T. Dinkova-Kostova and P. Talalay, *Carcinogenesis* **20,** 911 (1999).

[23] K. Singletary, C. MacDonald, M. Iovinelli, C. Fisher, and M. Wallig, *Carcinogenesis* **19,** 1039 (1998).

screen, and compare the potency of various plant sources in both plant breeding and molecular genetics applications. Highlights of some of these findings are described herein.

Many of the 9 recognized classes of phase 2 enzyme inducers were originally identified using the Prochaska assay.[7,11,24–26] These inducers include (see review[27]): (i) oxidizable *ortho*- and *para*-diphenols, their cognate quinones, and other Michael reaction acceptors; (ii) isothiocyanates[28] – highly electrophilic compounds that are widely consumed as their dietary precursor glucosinolates which are hydrolyzed to isothiocyanates by the enzyme myrosinase, which co-exists in plant cells and is present in the human gastrointestinal tract[29,30]; (iii) dithiocarbamates, which are formed metabolically by conjugation of GSH with isothiocyanates. Synthetic dithiocarbamates are widely used industrial and agricultural chemicals; (iv) 1,2 dithiole-3-thione derivatives such as oltipraz[19]; (v) trivalent arsenic derivatives such as sodium arsenite and phenylarsine oxide; (vi) divalent heavy metal derivatives; (vii) hydroperoxides such as *tert*-butyl hydroperoxide; (viii) polyenes such as carotenoid metabolites[11]; and (ix) vicinal dimercaptans such as 2,3-dimercaptopropanol. Synergism between compounds in certain of these classes has been demonstrated,[31] and whereas many of the inducers that have been isolated from natural products fit into one of these classes, others appear to involve novel chemistry. Compounds identified as inducers by means of the Prochaska assay include withanolides,[32,33] a bicyclic diarylheptanoid,[34] flavonoids,[35–38] and curcumin derivatives and other phenyl propenoids.[22,39–42] Furthermore, unpublished

[24] P. Talalay, M. J. De Long, and H. J. Prochaska, *Proc. Natl. Acad. Sci. USA* **85,** 8261 (1988).
[25] T. Prestera, W. D. Holtzclaw, Y. Zhang, and P. Talalay, *Proc. Natl. Acad. Sci. USA* **90,** 2965 (1993).
[26] P. Talalay, *Proc. Amer. Phil. Soc.* **143,** 52 (1999).
[27] P. Talalay, A. T. Dinkova-Kostova, and W. D. Holtzclaw, *Adv. Enzyme Regul.* **43,** 121 (2003).
[28] Y. Zhang, P. Talalay, C. G. Cho, and G. H. Posner, *Proc. Natl. Acad. Sci. USA* **89,** 2399 (1992).
[29] J. W. Fahey, A. T. Zalcmann, and P. Talalay, *Phytochemistry* **56,** 5 (2001); corrigendum *Phytochemistry* **59,** 237.
[30] T. A. Shapiro, J. W. Fahey, K. L. Wade, K. K. Stephenson, and P. Talalay, *Cancer Epidemiol. Biomarkers Prev*. **10,** 501 (2001).
[31] R. R. Putzer, Y. Zhang, T. Prestera, W. D. Holtzclaw, K. L. Wade, and P. Talalay, *Chem. Res. Toxicol.* **8,** 103 (1995).
[32] R. I. Misico, L. L. Song, A. S. Veleiro, A. M. Cirigliano, M. C. Tettamanzi, G. Burton, G. M. Bonetto, V. E. Nicotra, G. L. Silva, R. R. Gil, J. C. Oberti, A. D. Kinghorn, and J. M. Pezzuto, *J. Nat. Prod*. **65,** 677 (2002).
[33] E. J. Kennelly, C. Gerhäuser, L. L. Song, J. G. Graham, C. W. W. Beecher, J. M. Pezzuto, and A. D. Kinghorn, *J. Agric. Food Chem*. **45,** 3771 (1997).

work from our lab, and recent surveys[13,17,27] reveal a wide spectrum of inducers that includes both plant components and pharmaceutical agents. With curcumin, a naturally occurring spice component, Dinkova-Kostova[22,41] has used the Prochaska bioassay to elucidate which portions of the molecule are essential for inducer activity: synthetic as well as the naturally-occurring structural analogs were examined. These studies showed that the hydroxyl group had to be present at the *ortho*-position on the phenolic rings, and that the rings had to be bridged by a β-diketone moiety in order to be strong inducers. In studies with our colleague G.H. Posner, a number of synthetic norbornyl structural analogs of sulforaphane were examined, and one had equivalent inducer potency to sulforaphane in Hepa 1c1c7 cells.[17,18] Subsequently, Pezzuto and colleagues developed sulforamate, an analog of sulforaphane with comparable inducer potency and somewhat less toxicity.[12] This group has also developed 4′-bromoflavone as a derivative of naturally occurring flavonoids, guided by the Prochaska bioassay.[39]

Numerous reports now document the inducer potency of various plants, plant constituents, and complex dietary ingredients[10,28,30,35,43–64]

[34] D. S. Jang, E. J. Park, M. E. Hawthorne, J. S. Vigo, J. G. Graham, F. Cabieses, B. D. Santarsiero, A. D. Mesecar, H. H. Fong, R. G. Mehta, J. M. Pezzuto, and A. D. Kinghorn, *J. Agric. Food Chem*. **50,** 6330 (2002).
[35] J. W. Fahey and K. K. Stephenson, *J. Agric. Food Chem*. **50,** 7472 (2002).
[36] S. Yannai, A. J. Day, G. Williamson, and M. J. Rhodes, *Food Chem. Toxicol*. **36,** 623 (1998).
[37] Y. Uda, K. R. Price, G. Williamson, and M. J. Rhodes, *Cancer Lett*. **120,** 213 (1997).
[38] C. L. Miranda, G. L. Aponso, J. F. Stevens, M. L. Deinzer, and D. R. Buhler, *Cancer Lett*. **149,** 21 (2000).
[39] L. L. Song, J. W. Kosmeder, S. K. Lee, C. Gerhäuser, D. Lantvit, R. C. Moon, R. M. Moriarty, and J. M. Pezzuto, *Cancer Res*. **59,** 578 (1999).
[40] A. T. Dinkova-Kostova, C. Abeygunawardana, and P. Talalay, *J. Med. Chem*. **41,** 5287 (1998).
[41] A. T. Dinkova-Kostova, M. A. Massiah, R. E. Bozak, R. J. Hicks, and P. Talalay, *Proc. Natl. Acad. Sci. USA* **98,** 3404 (2001).
[42] A. T. Dinkova-Kostova and P. Talalay, *Free Radic. Biol. Med*. **29,** 231 (2000).
[43] N. Tawfiq, S. Wanigatunga, R. K. Heaney, S. R. Musk, G. Williamson, and G. R. Fenwick, *Eur. J. Cancer Prev*. **3,** 285 (1994).
[44] N. Tawfiq, R. K. Heaney, J. A. Plumb, G. R. Fenwick, S. R. Musk, and G. Williamson, *Carcinogenesis* **16,** 1191 (1995).
[45] T. A. Shapiro, J. W. Fahey, K. L. Wade, K. K. Stephenson, and P. Talalay, *Cancer Epidemiol. Biomarkers Prev*. **7,** 1091 (1998).
[46] K. Hashimoto, S. Kawamata, N. Usui, A. Tanaka, and Y. Uda, *Cancer Lett*. **180,** 1 (2002).
[47] M. W. Farnham, J. W. Fahey, and K. K. Stephenson, *J. Amer. Soc. Hort. Sci*. **125,** 482 (2000).
[48] M. W. Farnham, P. E. Wilson, K. K. Stephenson, and J. W. Fahey, *Plant Breeding* (in press).
[49] C. Y. Zhu and S. Loft, *Food Chem. Toxicol*. **41,** 455 (2003).
[50] F. M. Pereira, E. Rosa, J. W. Fahey, K. K. Stephenson, R. Carvalho, and A. Aires, *J. Agric. Food Chem*. **50,** 6239 (2002).

(see recent reviews by Pezzuto[65] and by Talalay[26]). Application of this assay to plant breeding, biochemistry, physiology, and molecular biology suggests that it may facilitate advances in the development of "tailored" vegetables and fruit that are rich inducers.[14–16,47,48,66,67]

Versatility and Limitations of The Prochaska Bioassay

Test Compound Matrix

The Prochaska bioassay, originally described for pure compounds, has been adapted to a wide range of materials including plants and plant extracts, urine, food (crude homogenates of dietary components), honey, and wine.

The application of this bioassay to plant extracts may now represent its most widespread use. In the original studies on plants in our laboratory, a wide variety of vegetables were tested for NQO1 inducer potency.[2] Those of the *Brassica* (e.g., broccoli) and *Allium* (e.g., onion) families showed inducer potency and various others had little or none.[2] This information led to the isolation of sulforaphane from broccoli,[28] and broccoli sprouts.[10] Subsequently, the assay has been used to guide the isolation of resveratrol from grapes,[54] withanolides from tomatillos and other plants,[32,33] isothiocyanates closely related to sulforaphane from wasabi and watercress,[61,66,67]

[51] J. W. Fahey and K. K. Stephenson, *HortScience* **34,** 1159 (1999).
[52] J. Bomser, D. L. Madhavi, K. Singletary, and M. A. Smith, *Planta Med*. **62,** 212 (1996).
[53] L. C. Chang, C. Gerhäuser, L. Song, N. R. Farnsworth, J. M. Pezzuto, and A. D. Kinghorn, *J. Nat. Prod*. **60,** 869 (1997).
[54] M. Jang, L. Cai, G. O. Udeani, K. V. Slowing, C. F. Thomas, C. W. Beecher, H. H. Fong, N. R. Farnsworth, A. D. Kinghorn, R. G. Mehta, R. C. Moon, and J. M. Pezzuto, *Science* **275,** 218 (1997).
[55] H. J. Park, Y. W. Lee, H. H. Park, Y. S. Lee, I. B. Kwon, and J. H. Yu, *Eur. J. Cancer Prev*. **7,** 465 (1998).
[56] K. J. Hintze, A. S. Keck, J. W. Finley, and E. H. Jeffery, *J. Nutr. Biochem*. **14,** 173 (2003).
[57] A. S. Keck, Q. Qiao, and E. H. Jeffery, *J. Agric. Food Chem*. **51,** 3320 (2003).
[58] N. V. Matusheski and E. H. Jeffery, *J. Agric. Food Chem*. **49,** 5743 (2001).
[59] C. Matito, F. Mastorakou, J. J. Centelles, J. L. Torres, and M. Cascante, *Eur. J. Nutr*. **42,** 43 (2003).
[60] S. K. Lee, L. Song, E. Mata-Greenwood, G. J. Kelloff, V. E. Steele, and J. M. Pezzuto, *Anticancer Res*. **19,** 35 (1999).
[61] P. Rose, K. Faulkner, G. Williamson, and R. Mithen, *Carcinogenesis* **21,** 1983 (2000).
[62] R. Mithen, K. Faulkner, R. Magrath, P. Rose, G. Williamson, and J. Marquez, *Theor. Appl. Genet*. **106,** 727 (2003).
[63] K. Faulkner, R. Mithen, and G. Williamson, *Carcinogenesis* **19,** 605 (1998).
[64] Y. H. Shon and K. S. Nam, *J. Ethnopharmacol*. **77,** 103 (2001).
[65] J. M. Pezzuto, *Biochem. Pharmacol*. **53,** 121 (1997).

various proanthocyanidins from blueberries,[52] and flavonoids from *Tephrosia* sp.,[53] onions,[68] buckwheat,[35] and Thai ginger.[35] Numerous other laboratories have reported on the inducer activity of various uncharacterized plant and fungal extracts including aqueous extracts of Brussels sprouts[49] and solvent extracts of 45 different plants.[46] The extraction protocol is critically important, and is addressed elsewhere in this chapter. Chemical structures of NQO1 inducers are reviewed in Chapter 23 of this volume.[69]

One novel dosing protocol utilizes plant leaf disc punches which are incubated in microtiter plates prepared essentially as described above. After a suitable incubation (leaching) period, the discs are removed, and the assay is performed.[14–16] The response is thus proportional to the amount of inducer leaching out into culture medium, and this, in turn, is proportional to cut surface area/volume. In this manner, several thousand chemically mutagenized *Arabidopsis* plants were screened for altered leaf NQO1 inducer potency and bioassay results were used as a guide for selective HPLC analysis of progeny from the putative mutants.[14–16]

We have used the Prochaska assay as a tool to assist us in verifying dietary compliance in clinical trials. For example, when human volunteers were fed various levels of glucosinolates and/or isothiocyanates following or preceding a period of prescribed abstinence from cruciferous vegetables (a source of NQO1-inducing isothiocyanates), we were able to verify compliance by bioassaying their urines for inducer activity during the control period. Likewise, we could confirm compliance with dietary intake of cruciferous vegetables. These results were entirely congruent with both the dietary record, and with an independent chemical test for the presence of isothiocyanate metabolites.[30] Also, in these clinical trials we first used the Prochaska bioassay to monitor dietary ingredients for unexpected phase 2 enzyme induction potency. Had there been significant levels of inducers in, for example, white bread, CocaCola™, green beans or tomatoes, we would have avoided administering these food products to subjects as part of a baseline diet. We were able to test these and all other dietary ingredients by appropriate dilution and homogenization of dietary components in order to develop a ''non-inducing'' baseline diet. In this case, there were no independent chemical tests that would have ruled out all possible sources of inducers.

[66] D. X. Hou, M. Fukuda, M. Fujii, and Y. Fuke, *Cancer Lett.* **161,** 195 (2000).
[67] D. X. Hou, M. Fukuda, M. Fujii, and Y. Fuke, *Int. J. Mol. Med.* **6,** 441 (2000).
[68] G. Williamson, G. W. Plumb, Y. Uda, K. R. Price, and M. J. Rhodes, *Carcinogenesis* **17,** 2385 (1996).
[69] A. T. Dinkova-Kostova, J. W. Fahey, and P. Talalay, *Meth. Enzymol.* **382,** 423 (2004).

We have also used this bioassay to compare the NQO1 inducing potential of various honeys and molasses.[35] We found that the induction potentials of honey and molasses were directly proportional to their color intensity (darkness), and it appeared that a number of flavones common to honey may have been responsible for this induction. Further analysis of the pure flavones then led us to identify very rich plant sources of these compounds.

Dosing

For assay of either pure chemicals, or plant extracts, a number of solvents including DMSO, dimethyl formamide, acetonitrile, ''triple solvent'' (a mixture of equal volumes of DMSO, dimethyl formamide and acetonitrile), tetrahydrofuran, ethanol, and methanol can be used to prepare the initial stock solution. In order to avoid cytotoxicity, the maximum tolerated concentration of these solvents for a 48-h induction period is 0.1% for tetrahydrofuran, 0.25% for dimethyl formamide, 0.33% for methanol and DMSO, 0.5% for triple solvent, and 1% for acetonitrile and ethanol. If examining the NQO1 inducer potential of urine or other aqueous solutions, one can add as much as about 10% of the volume of the microtiter plate well fluid.

The Prochaska bioassay presents unique opportunities for examining the kinetics of isothiocyanate effects on enzyme induction. Y. Zhang has shown that isothiocyanates are very rapidly absorbed by cultured cells,[70–74] and in a number of clinical studies they were shown to be rapidly metabolized.[30,45] Furthermore, some isothiocyanates (e.g., allyl isothiocyanate) are quite volatile, whereas others (e.g., sulforaphane) are not. Vapor phase transfer of inducers between nearby wells can complicate assays, even for compounds which are not usually considered to be especially volatile. An interesting example of vicinity effects among microtiter plate wells was encountered with dimethyl fumarate, which has been used previously as an inducer of NQO1.[24,75] This compound has very low volatility, yet when introduced into microtiter plate wells, cells in adjacent wells become induced. The mechanism of this effect is not understood.

[70] Y. Zhang and P. Talalay, *Cancer Res*. **58,** 4632 (1998).
[71] Y. Zhang, *Carcinogenesis* **21,** 1175 (2000).
[72] Y. Zhang, *Carcinogenesis* **22,** 425 (2001).
[73] L. Ye and Y. Zhang, *Carcinogenesis* **22,** 1987 (2001).
[74] Y. Zhang and E. C. Callaway, *Biochem. J*. **364,** 301 (2002).
[75] A. Begleiter, K. Sivananthan, T. J. Curphey, and R. P. Bird, *Cancer Epidemiol. Biomarkers Prev*. **12,** 566 (2003).

Equimolar concentrations of isothiocyanates compared to glucosinolates with added myrosinase (thioglucoside glucohydrolase; EC 3.2.3.1; the enzyme that catalyzes the conversion of glucosinolates to isothiocyanates) do not induce equally in the Prochaska bioassay. When an excess of highly purified myrosinase is added to each well (in the presence of ascorbate), complete hydrolysis of glucosinolates to their cognate isothiocyanates occurs *in-situ,* during the assay.[10] Interestingly, enzymatic release of isothiocyanates from glucosinolates during the assay gives higher inducer potencies than direct addition of the isothiocyanate alone. We have reproduced this situation with a number of glucosinolate/isothiocyanate pairs, using highly purified myrosinase.[76] (Neither the glucosinolate alone, nor myrosinase alone, induced NQO1). In all cases examined (data not shown), enzymatic release of isothiocyanate from its cognate glucosinolate was clearly more effective in inducing NQO1 than was direct addition of the isothiocyanate product.

Advantages of the Hepa 1c1c7 Cell Line

We have examined a variety of rodent, insect and human cell lines and found that the large amplitude of NQO1 response produced by Hepa 1c1c7 cells makes this line ideal for use in such a bioassay. Additionally, these cells maintain their responsiveness through a large number of passages, making standardization of the assay more reliable. Some inducers elevate NQO1 levels in Hepa 1c1c7 cells as high as 8- or 10-fold over the levels found in untreated control cells (Fig. 3; which also provides an example of the passage effect). NQO1 induction by some or all of these compounds, as well as by other standard inducers such as sulforaphane, have also been examined in the following: human gastric epithelial, AGS (Fig. 4); human laryngeal epithelial, HEp-2 (Fig. 4); human adult retinal pigmented epithelial, APRE-19 (Gao *et al.,*[77] and Fig. 4); human breast cancer, MCF7[41]; human skin keratinocyte, HaCaT[41,75]; murine leukemia L1210[77]; and murine keratinocyte, PE,[77] cell lines. In general, most cell lines were less sensitive to inducers than Hepa 1c1c7 cells. The commonly used invertebrate cell lines Sf9 (from *Spodoptera frugiperda*), Sf21 (also from *S. frugiperda*), and Tni (from *Trichoplusia ni*) did not respond even at the highest levels of several compounds tested (data not shown). Others have evaluated the use of cell lines such as a Chinese hamster ovary

[76] M. Shikita, J. W. Fahey, T. R. Golden, W. D. Holtzclaw, and P. Talalay, *Biochem. J.* **341,** 725 (1999).

[77] X. Gao, A. T. Dinkova-Kostova, and P. Talalay, *Proc. Natl. Acad. Sci. USA* **98,** 15221 (2001).

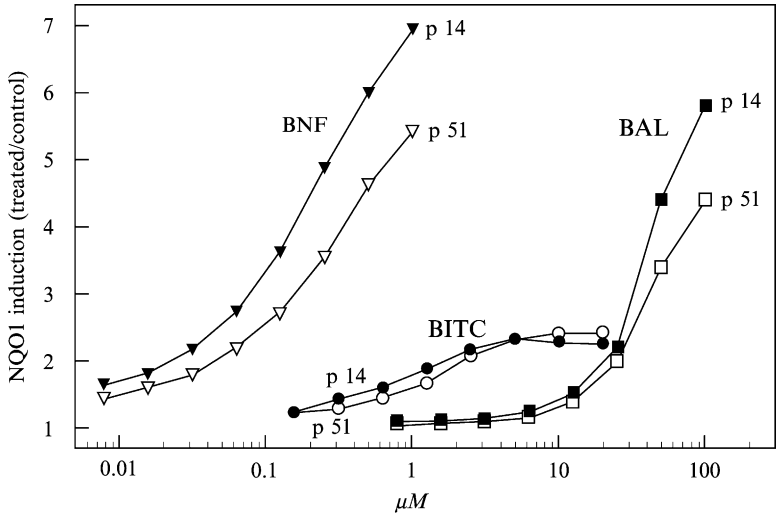

FIG. 3. Induction of NQO1 (treated/control NQO1 activity) following dosing with BNF (▼,▽), benzyl isothiocyanate (BITC; ●,○), or 2,3-dimercapto-1-propanol (British anti-Lewisite or BAL; ■,□). Filled symbols represent Hepa 1c1c7 cell line passage 14 and open symbols represent passage number 51. CD's for the early and late passages of BNF, BITC, and BAL, are 0.022, 0.044, 1.5, 2.2, 20, and 24 μM, respectively.

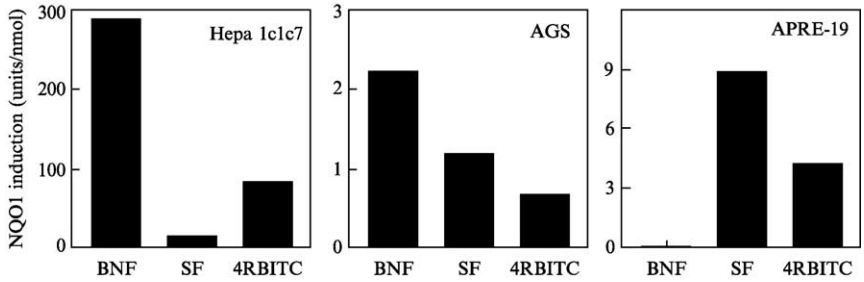

FIG. 4. Induction of NQO1 in Hepa 1c1c7, AGS, and APRE-19 cells treated for 48 h with BNF, sulforaphane (SF), or 4-(rhamnopyranosyloxy)benzyl isothiocyanate (4RBITC). Dosing of Hep-2 cells with the same three inducers yielded no induction with BNF, and produced substantial cytotoxicity with the 2 isothiocyanates such that a CD could not be measured.

(CHO) line which was transfected with high levels of human NQO1 in order to achieve over-expression of the enzyme.[78] Recently published work from Jiang and colleagues[79] has compared induction in Hepa 1c1c7 cells to induction in 6 other mammalian cell lines (MCF7, MDA-MB-231, HeLa, HT-29, HepG2, LNCaP) by using BNF and sulforaphane as standard inducers, and they have come to much the same conclusion – that the Hepa 1c1c7 cells "were the most robust and sensitive cells, which had higher basal as well as up-regulated enzymatic activities."

Because NQO1 induction in cells involves uptake, metabolism, and transcriptional events, it is not surprising that different cell types produce radically different induction profiles when presented with the same array of inducers. For example, human gastric and retinal epithelial cell lines respond very differently to treatment with two dissimilar isothiocyanates, than does the murine hepatoma cell line Hepa 1c1c7 (Fig. 4). Whether this is due to differences in uptake, metabolism, or signal processing, is not known, but comparative uptake kinetics of these and other isothiocyanates can now be examined. There is clearly a very broad variation of potencies among cell types. Thus NQO1 induction in Hepa 1c1c7 cells is as much as two orders of magnitude greater than in AGS cells. The relative NQO1 inducer potencies of two isothiocyanates were compared: 4-methylsulfinylbutyl isothiocyanate, (sulforaphane, from *Brassica oleracea* var. *italica* and 4-(rhamnopyranosyloxy)benzyl isothiocyanate from *Moringa oleifera*. In Hepa 1c1c7 cells, the NQO1 inducer potency of the *Moringa* isothiocyanate was many-fold higher than that of sulforaphane yet with both human cell types tested its potency was considerably lower than that sulforaphane (Fig. 4).

And finally, in Hepa 1c1c7 cells bifunctional inducers such as BNF,[24] induce phase 1 enzymes (e.g., cytochromes P-450), as well as NQO1, but they require activation by the cytochromes P-450 in order to exert inducer activity. Such activation is probably lacking in APRE-19 cells since BNF is not an inducer. This compound is one of the most potent inducers of phase 2 enzymes in Hepa 1c1c7 cells. The behavior of mutant Hepa 1c1c7 cell lines that are defective in either aryl hydrocarbon *(Ah)* receptor function (BP^rc1 cells) or aryl hydrocarbon hydroxylase expression (c1 cells), can be used to determine whether compounds are mono- or bifunctional inducers.[80] For example two isothiocyanates, sulforaphane and progoitrin

[78] L. H. De Haan, A. M. Boerboom, I. M. Rietjens, D. Van Capelle, A. J. De Ruijter, A. K. Jaiswal, and J. M. Aarts, *Biochem. Pharmacol.* **64,** 1597 (2002).
[79] Z. Q. Jiang, C. Chen, B. Yang, V. Hebbar, and A. N. Kong, *Life Sci.* **72,** 2243 (2003).
[80] H. J. Prochaska and P. Talalay, *Cancer Res.* **48,** 4776 (1988).

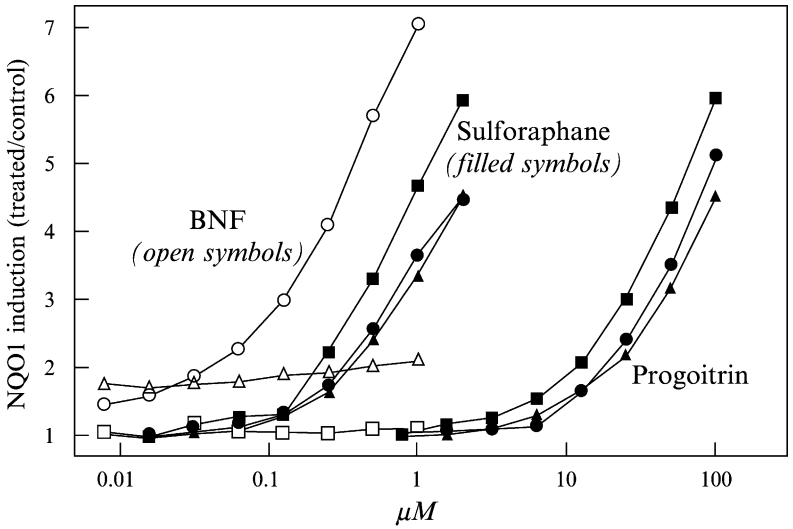

FIG. 5. NQO1 induction in Hepa 1c1c7 cells (●,○) and their mutants BP^{r}c1, defective in aryl hydrocarbon receptor function (▲,△), and c1, defective in aryl hydrocarbon hydroxylase expression (■,□). Cells were treated for 48 h with BNF, sulforaphane, or progoitrin. Only BNF required metabolic activation to induce NQO1.

(2-hydroxybut-3-enyl isothiocyanate), are fully active as inducers in wild type and mutant cell lines and are thus termed "monofunctional," whereas when BNF is tested against the same 3 cell types it does not induce NQO1 in cells lacking the cytochrome P450 (Fig. 5). These mutants have also been used to characterize flavonoids as mono- and bifunctional NQO1 inducers.[35,36]

Bioassay Variability

As is the case with all bioassays, and by the very nature of cell culture, there is inherent and unexplained variability in this bioassay. Certain investigators continue to use the 24 h dosing or exposure period originally suggested.[1] Most laboratories, however, use the 48 h induction period described in Prochaska *et al.*[2] and subsequent publications. Although cells should not be used after a certain number of passages (we usually retire sub-lines after 30 passages, but have gone as high as 45 or more on occasion), we have not detected an overall trend towards reduced sensitivity to inducers, although the absolute amplitude of the response (ratio of NQO1 levels in treated vs. control cells) may decline (Fig. 3). On close examination of the data obtained using BNF as a standard in all

assays over a period of 10 years in our laboratory (CD mean, 0.028 μM; SD, 0.015 μM; maximum, 0.082 μM; minimum, 0.015 μM; data from 253 assays performed by 5 different operators between 1993 and 2001), we can find no systematic pattern of variations relating to operator, operator training, cell history, season, culture medium components (source or lot), or any other identifiable extrinsic factor. Careful attention must be paid to timing, conditions, and cell culture status. Assay variability should be tracked and controlled by use of the same standard compound in all experiments (e.g., BNF).

Acknowledgments

We appreciate the patience and care of the following persons who, in addition to the authors, performed some of the bioassays described in this paper: Xiangqun Gao, Kristina Wade, Paula Wilson, Mark Wrona, and Yuesheng Zhang. We pay special tribute to Hans Prochaska *[deceased],* who along with Annette Santamaria, and Mary De Long, first developed this versatile assay. Hepa1c1c7 cells were originally provided by J. P. Whitlock, Jr.

[15] Structure-Activity Relationships in Two-Electron Reduction of Quinones

By NARIMANTAS ČĖNAS, ŽILVINAS ANUSEVIČIUS, HENRIKAS NIVINSKAS, LINA MISEVIČIENĖ, and JONAS ŠARLAUSKAS

Introduction

Quinones may accept electrons from various flavoenzymes, iron-sulfur proteins and photosynthetic reaction centers. The energetics of the quinone reduction have been studied extensively by pulse-radiolysis, electron spin resonance and electrochemical techniques. The studies of Yamazaki and coworkers in the early 1970s have shown that flavoenzymes may reduce quinones in single-electron, mixed single- and two-electron, and two-electron methods.[1,2] The single-electron reduction of quinones by flavoenzyme dehydrogenases-electrontransferases may be treated according to an ''outer-sphere electron transfer'' model[3–7] and is relatively well

[1] T. Iyanagi and I. Yamazaki, *Biochim. Biophys. Acta* **172,** 370 (1969).
[2] T. Iyanagi and I. Yamazaki, *Biochim. Biophys. Acta* **216,** 282 (1970).
[3] R. Marcus and N. Sutin, *Biochim. Biophys. Acta* **811,** 265 (1985).

0076-6879/04 $35.00