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FEBS Letters 580 (2006) 998–1009

Minireview

The molecular basis of multidrug resistance in cancer: The early years of P-glycoprotein research

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Received 2 December 2005; revised 15 December 2005; accepted 16 December 2005

Available online 28 December 2005

Edited by Gerrit van Meer

Abstract **The discovery and characterization of P-glycoprotein, an energy-dependent multidrug efflux pump, as a mechanism of multidrug resistance in cancer is generally accepted as a significant contribution to the ongoing effort to end death and suffering from this disease. The historical reflections of Victor Ling and Michael Gottesman concerning the early years of this research highlight the important contributions of the multidisciplinary teams involved in these studies, and illustrate how technological developments in biochemistry and molecular and cell biology enabled this discovery.**
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Keywords: Multidrug resistance; P-glycoprotein; MDR1; Somatic cell genetics; Transport; Chemotherapy

1. Introduction

The first successful chemotherapy of human cancer led soon to the realization that drug resistance was going to be a major impediment to cure or long-term palliation. The simple expedient of employing multiple cytotoxic drugs with different mechanisms of action was not the solution to this problem, since cancers appeared able to develop resistance simultaneously to many different anti-cancer drugs. So the search began for mechanisms that could account for this multidrug resistance (MDR) in various cultured cell models, and the search is still on for which of these mechanisms and/or mechanisms as yet undiscovered are clinically relevant targets whose circumvention can improve cancer therapy.

Advances in understanding MDR in cancer went hand-in-hand with development of research tools including the marriage of biochemistry, molecular biology, somatic cell genetics, and cell biology that revolutionized all of modern biomedical research. Table 1 summarizes some of the discoveries about P-glycoproteins (MDR1 and MDR2(3)) that resulted from this research. The early story of how these tools were applied to gain insight into cancer MDR, told from the personal point of view of the writers of this review, is both a reflection of multiple intellectual inputs from the several groups that were working on this problem, and the exploitation of state-of-the-art approaches becoming available as the work was being done.

2. The cellular physiology and biochemistry of multidrug resistance (1968–1986): Victor Ling and colleagues

After my post-doctoral training in Cambridge with Fred Sanger in 1971, I returned to Toronto to set up my own laboratory at the Ontario Cancer Institute. I wanted to be involved in the exciting somatic cell genetics program that Lou Siminovitch, Jim Till, and Gordon Whitmore had set up. I was particularly impressed by an earlier publication from that group showing that it was possible to isolate temperature-sensitive mutants in mouse L-cells [1]. Although the nature of the defect was not known at the time, it was eventually localized to a protein involved in DNA topoisomerase II activity [2]. By the time I arrived, it had pretty much been decided that the Chinese hamster ovary (CHO) cells were the cell line of choice. The reasons were that CHO cells maintained a relatively stable and near normal karyotype in culture, were able to grow in monolayer with a high cloning efficiency (>50%), could grow in suspension for mass production, could be easily synchronized for cell-cycle studies, and importantly, stable mutants and temperature-sensitive mutants could be isolated [3,4].

Larry Thompson and Bud Baker were most generous in "showing me the ropes" of how to isolate clones, how to maintain mammalian cells in culture, etc. It was team science at its best. The overarching philosophy of the Toronto somatic cell genetics group was that the investigation of complex biological phenomena was best approached by exploiting the power of genetics – to identify phenotypes that were genetically linked as opposed to those that were not. A key issue at the time was to determine whether or not an observed variant phenotype was due to a genuine mutation (which could be stably inherited and used as a genetic marker). In that context, protocols for isolating variants with freshly cloned cell populations using single-step selections were developed in order to maximize the probability of obtaining variants resulting from single mutations. The same principle was used for isolating revertants. Luria–Delbrück fluctuation analyses were undertaken to determine if the appearance of variants was consistent with spontaneous mutation rates. The use of mutagens result-

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0014-5793/$32.00 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2005.12.060

Table 1
Timeline of significant discoveries related to MDR1 and MDR2(MDR3)

Date	Discovery	Reference
1968	Isolation of MDR Chinese hamster cell lines	Kessel, D., Botterill, V. and Wodinsky, I. Cancer Res. 28, 938–941
1970	Demonstration of cytogenetic abnormalities in MDR cells consistent with gene amplification	Biedler, J.L. and Riehm, H. Cancer Res. 30, 1174–1184
1973	Demonstration of increased drug efflux in MDR cell lines causing decreased drug accumulation	Danø, K. Biochem. Biophys. Acta 323, 466–483
1973	Demonstration of inhibition of drug efflux causing increased drug accumulation	Danø, K. op. cit.
1974	Isolation of a series of related colchicine resistant CHO clonal cell lines using single step selections; Increasing colchicine resistance correlated with resistance to other drugs and reduced drug uptake	Ling, V. and Thompson, L.H. J. Cell. Physiol. 83, 103–116
1974	Isolation of single-step revertants showing reversion of MDR phenotype	Ling, V. Can. J. Genet. Cytol. 17, 503–515
1974	Evidence that drug resistance is energy-dependent	See, Y.P., Carlsen, S.A., Till, J.E. and Ling, V. Biochem. Biophys. Acta. 373, 242–252
1976	Demonstration of P-glycoprotein expression in MDR cell lines	Juliano, R.L. and Ling, V. Biochim. Biophys. Acta 455, 152–162
1978	MDR shown to be a dominant phenotype	Ling, V. and Baker, R.M. Somatic Cell Genet. 4, 193–200
1979	First purification of P-gp	Riordan, J.R. and Ling, V. J. Biol. Chem. 254, 12701–12705
1979	Isolation of MDR human cancer cell lines	Beck, W.T., Mueller, T.J. and Tanzer, L.R. Cancer Res. 39, 2070–2076
1981	Demonstration that verapamil could reverse MDR indicated the possibility of clinically useful reversing agents for MDR	Tsuruo, T., Iida, H., Tsukagoshi, S. and Sakurai, Y. Cancer Res. 41, 1967–1972
1982	Mechanism of calcium-influx blockers and calmodulin inhibitors for overcoming resistance is to inhibit drug efflux and increase VCR and ADM accumulation	Tsuruo, T., Iida, H., Tsukagoshi, S. and Sakurai, Y. Cancer Res. 42, 4730–4733
1982	Confirmed dominant phenotype by gene transfer and the DNA transfer suggested a single or small number of linked genes	Debenham, P.G., Kartner, N., Siminovitch, L., Riordan, J.R. and Ling, V. Mol. Cell. Biol. 2, 881–889
1983	Various calcium influx blockers, including verapamil, can potentiate VCR and ADM effects	Tsuruo, T., Iida, H., Nojiri, M., Tsukagoshi, S. and Sakurai, Y. Cancer Res. 43, 2905–2910
1983	First indication that Pgp is commonly expressed in a variety of MDR cell lines using Western blots	Kartner, N., Riordan, J.R. and Ling, V. Science 221, 1285–1288
1984	Linking of P-gp and gene transfection and gene amplification	Robertson, S.M., Ling, V. and Stanners, C.P. Mol. Cell. Biol. 4, 500–506
1984	Quinidine and related compounds also have circumvented drug resistance	Tsuruo, T., Iida, H., Kitatani, Y., Yokota, K., Tsukagoshi, S. and Sakurai, Y. Cancer Res. 44, 4303–4307
1984	Demonstration and cloning of common amplified gene fragments in Chinese hamster MDR cell lines selected with different drugs	Roninson, I.B., Abelson, H.T., Housman, D.E., Howell, N. and Varshavsky, A. Nature 309, 626–628
1985	Detection of P-gp in clinical samples	Bell, D.R., Gerlach, J.H., Kartner, N., Buick, R.N. and Ling, V. J. Clin. Oncol. 3, 311–315
1985	Amplification of P-gp genes in multidrug-resistant mammalian cell lines	Riordan, J.R., Deuchars, K., Kartner, N., Alon, N., Trent, J. and Ling, V. Nature 316, 817–819
1985	Isolation of monoclonal antibodies (C219, C494) to P-glycoprotein	Kartner, N., Evernden-Porelle, D., Bradley, G. and Ling, V. Nature 316, 820–823
1985	ABC cassette superfamily defined	Higgins, C.F., Hiles, I.D., Whalley, K. and Jamieson, D.J. EMBO J. 4, 1033–1039
1986	Description of the P-gp/Mdr amplicon	Van der Blick, A.M., Van der Velde-Koerts, T., Ling, V. and Borst, P. Mol. Cell. Biol. 6, 1671–1678
1986	Detection of *MDR*1 and *MDR*2(*MDR*3) genes amplified in human cancer cells and cloning of the first human mdr sequences	Roninson, I.B., Chin, J.E., Choi, K., Gros, P., Housman, D.E., Fojo, A., Shen, D.-W., Gottesman, M.M. and Pastan, I. Proc. Natl. Acad. Sci. USA 83, 4538–4552
1986	Demonstration of 4.5 kb MDR1 mRNA message in human cell lines of increasing drug resistance	Shen, D.W., Fojo, A., Chin, J.E., Roninson, I.B., Richert, N., Pastan, I. and Gottesman, M.M. Science 232, 643–645
1986	Isolation of P-gp1 probe for overexpressed mRNAs in Chinese hamster cells	de Bruijn, M.H., Van der Bliek, A.M., Biedler, J.L. and Borst, P. Mol. Cell. Biol. 6, 4717–4722 Scotto, K.W., Biedler, J.L. and Melera, P.W. Science 232, 751–755
1986	Cloning of full-length MDR1 gene, demonstration of its homology to bacterial ATP-dependent transporters, and overall structure	Gros, P., Croop, J. and Housman, D.E. Cell 47, 371–390 Chen, C.-J., Chin, J.E., Ueda, K., Clark, D., Pastan, I., Gottesman, M.M. and Roninson, I.B. Cell 47, 381–389
1986	Cloning of the first mdr coding sequence and demonstration of ∼5 kb mdr mRNA message in multidrug-resistant hamster cell lines	Gros, P., Croop, J., Roninson, I., Varshavsky, A. and Housman, D.E. Proc. Natl. Acad. Sci. USA 83, 337–341
1986	Model of P-gp as an export pump by analogy to HlyB	Gerlach, J.H., Endicott, J.A., Juranka, P.F., Henderson, G., Sarangi, F., Deuchars K.L. and Ling, V. Nature 324, 485–489
1986	MDR1 encodes P-glycoprotein	Ueda, K., Cornwell, M.M., Gottesman, M.M., Pastan, I., Roninson, I.B., Ling, V. and Riordan, J.R. Biochem. Biophys. Res. Commun. 141, 956–962
1986	Full-length MDR1 cDNA confers multidrug resistance	Gros, P., Ben-Neriah, Y., Croop, J.M. and Housman, D.E. Nature 323, 728–731
1986	Anti-P-gp MoAb MRK-16, to an external epitope, reverses drug resistance	Hamada, H. and Tsuruo, T. Proc. Natl. Acad. Sci. USA 83, 7785–7789

(*continued on next page*)

Table 1 (*continued*)

Date	Discovery	Reference
1986	Membranes containing P-glycoprotein bind drugs (e.g., vinblastine)	Cornwell, M.M., Gottesman, M.M. and Pastan, I. J. Biol. Chem. 262, 7921–7928
1986	Photoaffinity labeling of P-gp	Cornwell, M.M., Safa, A.R., Felsted, R.L., Gottesman, M.M. and Pastan, I. Proc. Natl. Acad. Sci. USA 83, 3847–3851
1987	Expression pattern of MDR mRNA in normal tissues and human cancers	Fojo, A.T., Ueda, K., Slamon, D.J., Poplack, D.G., Gottesman, M.M. and Pastan, I. Proc. Natl. Acad. Sci. USA 84, 265–269
1987	P-gp is phosphorylated by C-kinase. The phosphorylation is inhibited by verapamil and trifluoperazine and is enhanced by C-kinase activator	Hamada, H., Hagiwara, K.I., Nakajima, T. and Tsuruo, T. Cancer Res. 47, 2860–2865
1987	MDR1 is a real gene, mainly expressed in the liver	Van der Bliek, A.M., Baas, F., Ten Houte de Lange, T., Kooiman, P.M., Van der Velde-Koerts, T. and Borst, P. EMBO J. 6, 3325–3331
1987	Expression pattern of P-gp in normal tissue by immunohistochemistry	Thiebaut, F., Tsuruo, T., Hamada, H., Gottesman, M.M., Pastan, I. and Willingham, M.C. Proc. Natl. Acad. Sci. USA 84, 7735–7738
1987	Identification of human MDR1 promoter	Ueda, K., Pastan, I. and Gottesman, M.M. J. Biol. Chem. 262, 17432–17436
1988	Explains why P-gp was not detected using boiled samples on Laemmli gels because of the unusual nature of P-gp	Greenberger, L.M., Scott, W.S., Georges, E., Ling, V. and Horwitz, S.B. J. Natl. Cancer Inst. 80, 506–510
1988	P-gp expression in normal tissue revealed; Positive tissues are adrenal cortex and medulla, renal tubules and placenta	Sugawara, I., Kataoka, I., Morishita, Y., Hamada, H., Tsuruo, T., Itoyama, S. and Mori, S. Cancer Res. 48, 1926–1929
1988	Point mutations in MDR1 change substrate specificity	Choi, K., Chen, C.-J., Kreigler, M. and Roninson, I.B. Cell 53, 519–529
1988	Retroviral vectors encoding MDR1 can confer MDR whose expression is apical in polarized epithelium	Pastan, I., Gottesman, M.M., Ueda, K., Lovelace, E., Rutherford, A.V. and Willingham, M.C. Proc. Natl. Acad. Sci. USA 85, 4486–4490
1988	Use of MDR1 vectors for co-transfection and selection of unselected genes	Kane, S.E., Troen, B.R., Gal, S., Ueda, K., Pastan, I. and Gottesman, M.M. Mol. Cell. Biol. 8, 3316–3321
1988	Transport of drugs by P-gp in isolated vesicles	Horio, M., Gottesman, M.M. and Pastan, I. Proc. Natl. Acad. Sci. USA 85, 3580–3584
1988	P-gp was homogenously purified by affinity chromatography using MRK-16. Purified P-gp has an ATPase activity	Hamada, H. and Tsuruo, T. J. Biol. Chem. 263, 1454–1458
1989	P-gp is expressed in brain capillaries	Thiebaut, F., Tsuruo, T., Hamada, H., Gottesman, M.M., Pastan, I. and Willingham, M.C. J. Histochem. Cytochem. 37, 159–164
1989	MDR-reversal mechanism of verapamil clarified; Verapamil actually binds to P-gp and can be transported by P-gp	Yusa, K. and Tsuruo, T. Cancer Res. 49, 5002–5006
1989	Mice expressing MDR1 in bone marrow are drug-resistant	Galski, H., Sullivan, M., Willingham, M.C., Chin, K.-V., Gottesman, M.M., Pastan, I. and Merlino, G.T. Mol. Cell. Biol. 9, 4357–4363
1990	Detection of P-gp isoforms by gene specific monoclonal antibodies	Georges, E., Bradley, G., Gariepy, J. and Ling, V. Proc. Natl. Acad. Sci. USA 87, 152–156
1990	Reverse-transcription PCR analysis demonstrates widespread MDR1 expression in human cancers	Noonan, K.E., Beck, C., Holzmayer, T.A., Chin, J.E., Wunder, J.S., Andrulis, I.L., Gazdar, A.F., Willman, C.L., Griffith, B., Von Hoff, D.D. and Roninson, I.B. Proc. Natl. Acad. Sci. USA 87, 7160–7164
1990	Genetic demonstration of distinct P-gp sites responsible for drug binding and release	Safa, A.R., Stern, R.K., Choi, K., Agresti, M., Tamai, I., Mehta, N.D. and Roninson, I.B. Proc. Natl. Acad. Sci. USA 87, 7225–7229
1990	Full-length sequence of mouse mdr2(mdr3) gene	Devault, A. and Gros, P. Mol. Cell. Biol. 10, 1652–1663
1990	Hormone (progesterone)-regulated expression of P-gp	Arceci, R.J., Baas, F., Raponi, R., Horwitz, S.B., Housman, D. and Croop, J.M. Mol. Reprod. Dev. 25, 101–109
1990	ATPase activity of P-gp	Germann, U.A., Willingham, M.C., Pastan, I. and Gottesman, M.M. Biochemistry 29, 2295–2303
1990	Hydrophobic vacuum cleaner model	Raviv, Y., Pollard, H.B., Bruggemann, E.P., Pastan, I. and Gottesman, M.M. J. Biol. Chem. 265, 3975–3980
1990	MDR-like genes in model organisms (e.g., L. tarentolae)	Ouellette, M., Fase-Fowler, F. and Borst, P. EMBO J. 9, 1027–1033
1991	Correlation of P-gp with outcome in children's cancer	Chan, H.S.L., Haddad, G., Thorner, P.S., DeBoer, G., Lin, Y.P., Ondrusek, N., Yeger, H. and Ling, V. N. Engl. J. Med. 325, 1608–1614
1991	MDR-like genes in model organisms (e.g., *C. elegans*, *D. melanogaster*)	Wu, C.T., Budding, M., Griffin, M.S. and Croop, J.M. Mol. Cell. Biol. 11, 3940–3948 Schinkel, A.H. and Borst, P. Semin. Cancer Biol. 2, 213–226
1991	P-gp expression in bone marrow stem cells explains the "side population" effect seen in these cells with fluorescent dyes	Chaudhary, P.M. and Roninson, I.B. Cell 66, 85–94
1992	Drug-stimulated ATPase of reconstituted P-gp; Vanadate inhibition	Ambudkar, S.V., Lelong, I.H., Zhang, J., Cardarelli, C.O., Gottesman, M.M. and Pastan, I. Proc. Natl. Acad. Sci. USA 89, 8472–8476
1992	P-gp is directly involved in blood–brain barrier	Tatsuta, T., Naito, M., Ohhara, T., Sugawara, I. and Tsuruo, T. J. Biol. Chem. 267, 20383–20391

Table 1 (*continued*)

Date	Discovery	Reference
1992	Retroviral transfer of MDR1 into bone marrow confers in vivo drug resistance	Sorrentino, B.P., Brandt, S.J., Bodine, D., Gottesman, M.M., Pastan, I., Cline, A. and Nienhuis, A.W. Science 257, 99–103
1993	Epigenetic induction of MDR1 expression by transient exposure to different chemotherapeutic drugs	Chaudhary, P.M. and Roninson, I.B. J. Natl. Cancer Inst. 85, 632–639
1993	MDR2 shown to be essential for transport of phosphatidylcholine in liver	Smit, J.J., Schinkel, A.H., Oude Elferink, R.P., Groen, A.K., Wagenaar, E., van Deemter, L., Mol, C.A., Ottenhoff, R., van der Lugt, N.M. and van Roon, M.A., et al. Cell 75, 451–462
1994	MDR1 knock out mouse has blood–brain barrier defects and alterations in pharmacokinetics of drugs	Schinkel, A.H., Smit, J.J., van Tellingen, O., Beijnen, J.H., Wagenaar, E., van Deemter, L., Mol, C.A., van der Valk, M.A., Robanus-Maandag, E.C. and te Riele, H.P., et al. Cell 77, 491–502
1994	Purification and reconstitution showing that P-gp can act alone to transport drugs	Shapiro, A.B. and Ling, V. J. Biol. Chem. 269, 3745–3754 Shapiro, A. and Ling, V. (1995) J. Biol. Chem. 270, 16167–16175
1995	Use of orthovanadate to trap P-gp in the post-hydrolysis transition state	Urbatsch, I.L., Sankaran, B., Weber, J. and Senior, A.E. J. Biol. Chem. 270, 19383–19390
1996	Clinical use of a reversing agent	Chan, H.S.L., DeBoer, D., Thiessen, J.T., Kingston, J.E., O'Brien, J.M., Koren, G., Giesnrecht, E., Haddad, G., Verjee, Z., Hungerford, J.L., Ling, V. and Gallie, B.L. Clin. Cancer Res. 2, 1499–1508
1996	Evidence that MDR2(MDR3) confers drug resistance	Kino, K., Taguchi, Y., Yamada, K., Komano, T. and Ueda, K. FEBS Lett. 399, 29–32
1996	Use of a "cys-less" mutant of P-gp and crosslinking agents to map the drug binding sites	Loo, T.W. and Clarke, D.M. J. Biol. Chem. 271, 27482–27487
1997	Use of 99m Tc-sestamibi uptake into tumors as a surrogate marker for P-gp function detectable by whole body imaging	Luker, G.D., Facasso, P.M., Dobkin, J. and Piwnica-Worms, D. J. Nucl. Med. 38, 369–372
1997	Evidence that there are at least two and probably more drug-binding sites on P-gp	Dey, S., Ramachandra, M., Pastan, I., Gottesman, M.M. and Ambudkar, S.V. Proc. Natl. Acad. Sci. USA 94, 10594–10599 Shapiro, A.B. and Ling, V. Eur. J. Biochem. 250, 130–137
1997	Gene rearrangement as a novel mechanism for MDR1 gene activation	Mickley, L.A., Spengler, B.A., Knutsen, T.A., Biedler, J.L. and Fojo, T.J. Clin. Invest. 99, 1947–1957
1997	Drug-transporting P-gp is not essential in metabolism, as long as mice are kept away from xenotoxins	Schinkel, A.H., Mayer, U., Wagenaar, E., Mol, C.A., van Deemter, L., Smit, J.J., van der Valk, M.A., Voordouw, A.C., Spits, H., van Tellingen, O., Zijlmans, J.M., Fibbe, W.E. and Borst, P. Proc. Natl. Acad. Sci. USA 94, 4028–4033
1997	P-gp is a major factor in reducing the oral availability of amphipathic drugs, as exemplified by Taxol	Sparreboom, A., van Asperen, J., Mayer, U., Schinkel, A.H., Smit, J.W., Meijer, D.K., Borst, P., Nooijen, W.J., Beijnen, J.H. and van Tellingen, O. Proc. Natl. Acad. Sci. USA 94, 2031–2035 Mechetner, E.B., Schott, B., Morse, B.S., Stein, W.D., Druley, T., Davis, K., Tsuruo, T. and Roninson, I.B. Proc. Natl. Acad. Sci. USA 94, 12908–12913
1997	P-gp has multiple drug binding sites that interact with each other	Shapiro, A.B. and Ling, V. Eur. J. Biochem. 250, 130–137
1997	P-gp recognizes drugs from the inner leaflet consistent with a hydrophobic vacuum model	Shapiro, A.B. and Ling, V. Eur. J. Biochem. 250, 122–129
1998	Demonstration that P-gp inhibits apoptosis through a mechanism unrelated to drug efflux in lymphoid cells	Smyth, M.J., Krasovskis, E., Sutton, V.R. and Johnstone, R.W. Proc. Natl. Acad. Sci. USA 95, 7024–7029
1998	Mechanism of action: cooperativity between two ATP sites in MDR1	Senior, A.E. and Bhagat, S. Biochemistry 37, 831–836
1998	Mechanism of action: P-gp shows reduced affinity for photoaffinity analog of drug-substrate in the Vi trapped transition state: a surrogate assay to monitor conversion of high affinity "ON" site to a low affinity "OFF" site	Ramachandra, M., Ambudkar, S.V., Chen, D., Hrycyna, C.A., Dey, S., Gottesman, M.M. and Pastan, I. Biochemistry 37, 5010–5019
1999	Use of inhibition of rhodamine 123 efflux as a surrogate assay for P-gp inhibition in CD56 peripheral mononucleate cells in clinical trials	Robey, R., Bakke, S., Stein, W., Meadows, B., Litman, T., Patil, S., Smith, T., Fojo, T. and Bates, S. Blood 93, 306–314
1999	Proposal that P-gp mediated ATP hydrolysis could be used both for transport and to 'reset' the molecule	Hrycyna, C.A., Ramachandra, M., Germann, U.A., Cheng, P.W., Pastan, I. and Gottesman, M.M. Biochemistry 38, 13887–13899
2000	MDR2(MDR3) gene can confer resistance to real anti-cancer drugs	Smith, A.J., van Helvoort, A., van Meer, G., Szabo, K., Welker, E., Szakacs, G., Varadi, A., Sarkadi, B. and Borst, P. J. Biol. Chem. 275, 23530–23539
2000	Mechanism of action: ATP hydrolysis needed to power transport and restore ability of P-gp molecule to bind substrate	Sauna, Z.E. and Ambudkar, S.V. Proc. Natl. Acad. Sci. USA 97, 2515–2520
2001	Mechanism of action: drug transport is driven by ATP binding while ATP hydrolysis resets the molecule	Rosenberg, M.F., Velarde, G., Ford, R.C., Martin, C., Berridge, G., Kerr, I.D., Callaghan, R., Schmidlin, A., Wooding, C., Linton, K.J. and Higgins, C.F. EMBO J. 20, 5615–5625
2001	Clinical trial showing survival advantage for patients with AML treated with MDR inhibition plus chemotherapy	List, A.F., Kopecky, K.J., Willman, C.L., Head, D.R., Persons, D.L., Slovak, M.L., Dorr, R., Karanes, C., Hynes, H.E., Doroshow, J.H., Shurafa, M. and Appelbaum, F.R. Blood 98, 3212–3220

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Table 1 (*continued*)

Date	Discovery	Reference
2002	Use of an MDR1 vector to force expression of a non-selectable co-transfected gene in vivo	Licht, T., Haskins, M., Henthorn, P., Kleiman, S.E., Bodine, D.M., Whitwam, T., Puck, J.M., Gottesman, M.M. and Melniczek, J.R. Proc. Natl. Acad. Sci. USA 99, 3123–3128
2004	Global prediction of MDR1 substrates, inhibitors and collateral sensitizers	Szakacs, G., Annereau, J.P., Lababidi, S., Shankavaram, U., Arciello, A., Bussey, K.J., Reinhold, W., Guo, Y., Kruh, G., Reimers, M., Weinstein, J.N. and Gottesman, M.M. Cancer Cell 6, 129–137

ing in increased frequency of variants was additional evidence for a genetic basis for the variant phenotype. Cell–cell hybrids were used to determine if a phenotype was dominant or recessive. The transfer of a variant phenotype to recipient cells using chromosome transfer or DNA transfection was additional evidence that the variant phenotype had a genetic basis. The colchicine-resistant cell lines in Chinese hamster ovary (CHO) cells were developed in that milieu [5,6].

The original purpose for isolating variant cell lines resistant to colchicine was to obtain mutants with altered colchicine-binding protein (tubulin) as an initial approach towards creating a class of cell division mutants. Clonal selections were undertaken in single steps without mutagen to minimize multiple changes. Higher levels of colchicine resistance were selected from clonal populations of lower resistance. Mutagen was used in some of these steps in order to increase the frequency and variety of variants obtained. Surprising to us was that of more than 20 colchicine-resistant clonal lines characterized, all had a pleiotropic cross-resistance to unrelated drugs such as daunomycin and puromycin, drugs not known to interact with microtubules, and all had reduced colchicine uptake into whole cells proportionate to the degree of drug resistance while the colchicine-binding ability of the mutant cell extracts was not significantly reduced. We concluded from this study that, "*colchicine resistance most probably stems from alteration(s) in the cell resulting in reduced permeability* [5]".

Unbeknown to us, after we submitted our CH^R mutant paper [5], Keld Danø submitted his elegant study of the kinetics of daunomycin uptake in resistant mouse Ehrlich ascites tumor cells [7]. He showed that the accumulation of daunomycin reached a lower steady-state in the resistant cells compared with the wild-type cells while the uptake into the isolated nuclei from these cells was similar. Moreover, the steady-state of labeled daunomycin increased in the presence of metabolic inhibitors such as 2-deoxyglucose, and also in the presence of structural analogs such as *N*-acetyldaunomycin. These observations along with the fact that the accumulated steady-state level of daunomycin increased in the presence of *Vinca alkaloids*, to which the tumor cells were cross-resistant, led Danø to conclude that the reduced accumulation of the drugs must be due to an energy-dependent carrier-mediated extrusion mechanism and that the *V. alkaloids* competed with daunomycin for the extrusion mechanism.

Y.P. See, a post-doctoral fellow in my lab, had also examined the energy dependence of colchicine accumulation in the CH^R cell lines [8]. He observed that in the absence of glucose a number of metabolic inhibitors such as azide and cyanide greatly stimulated the rate of colchicine uptake (up to 100-fold) in the CH^R cells. This was also observed with unrelated drugs (e.g., actinomycin D, and puromycin) and this increased uptake could be rapidly reversed by the addition of metabolizable sugars such glucose or ribose [8]. We speculated and later confirmed that these rapid drug uptake changes correlated with the rapid changes in the level of ATP in the cell [9]. Although our results were consistent with the conclusions of Danø, we felt at the time that the highly pleiotropic nature [10] of the MDR phenotype (e.g., cross-resistance to unrelated drugs and increased or collateral sensitivity to many membrane active agents) could not be explained easily by a classical drug efflux mechanism. We found it difficult to envision how a single transporter could recognize so many different compounds! At the same time, our study of initial rates of colchicine uptake under different conditions provided evidence that colchicine enters cells via an unmediated diffusion process. For example, we could not compete for colchicine uptake with the close structural analog colcemid. Also "fluidizers" of the membrane lipid bilayer such as non-ionic detergents and local anesthetics were able to stimulate drug uptake and reverse the drug resistance phenotype [5,9]. Moreover, when we measured the activation energies of colchicine uptake using Arrhenius plots in wild-type CHO cells in the presence of potassium cyanide (stimulated rate) or in its absence (normal rate), they were similar at 20 and 19 kcal per mole, respectively [11], suggesting that the mechanism of colchicine uptake, at least in wild-type cells, was similar in the presence or absence of the energy-dependent mechanism.

All this suggested to us that a more global effect on the cell membrane must be postulated in order to accommodate the MDR phenotype and we felt this would be more consistent with an "active" permeability barrier model. We suggested that a "modulator" of membrane lipid fluidity, perhaps involving phosphorylation/dephosphorylation of membrane proteins, could be the basis of such a model [6,9]. This model stimulated a lot of discussion and in a membrane meeting David Goldman's question was particularly insightful [11]. David raised the question of a carrier-mediated mechanism after some other queries:

Alper: *If you are using the same carrier for colchicine and other things, would you not expect to find competition if you look, say at colchicine uptake and then use actinomycin at the same time?*
Ling: *We looked at this closely and could find no competition between colchicine and puromycin, for example.*

....

Goldman: *Could you not have a carrier system with a very high Km?*
Ling: *One could. You would also have to postulate that this carrier is located on the membrane and that this carrier has specificity for a wide variety of compounds.*"

Twenty years later Adam Shapiro from my lab showed that P-glycoprotein (P-gp) recognizes its substrates from the lipid bilayer of the plasma membrane, from the inner leaflet, and since these substrates are hydrophobic in nature, they would

partition into the bilayer at very high concentrations and that the Km for P-gp transport of these compounds could well be very high! [12,13]. This also forms the basis for the ''hydrophobic vacuum'' model of P-gp [14,15].

While the characterization of the MDR phenotype was ongoing we continued to investigate the genetic relationship between the CH^R and the MDR phenotype. It should be noted that by the early 1970s a number of cell lines and transplantable tumors selected for resistance to anti-cancer drugs and having reduced drug uptake had been reported by various investigators (for example, Refs. [7,16–18]). These were not clonal lines nor were they selected with the goal of minimizing genetic changes, so multiple genetic changes were possible. We reasoned that if there were multiple mechanisms for the MDR phenotype, a variety of well-characterized independent isolates of CH^R mutants would be useful for delineating the different mechanisms [8]. However, further genetic characterization convinced us that the MDR phenotype was intimately linked to colchicine resistance (CH^R) and likely the result of a single gene alteration (or at least due to closely linked genes). In addition to the independently selected CH^R clones that all displayed the MDR phenotype, cell: cell hybrid studies showed that the MDR phenotype paralleled the CH^R phenotype in being expressed in an incompletely dominant fashion [5,19]. Moreover, independent revertant clones selected in a single step from the two highly resistant lines (CH^R C5 and CH^R C4) using tritiated leucine to kill puromycin-resistant CH^R cells which incorporated the labeled leucine in the presence of puromycin while the revertant puromycin-sensitive cells did not, also showed that the MDR and the CH^R phenotype both co-reverted [6]. Finally, when cellular DNA transfer technology using calcium phosphate-precipitated DNA was developed, [20], we showed that genomic DNA from cells transfected into drug-sensitive mouse L-cells resulted in the expression of both the CH^R and MDR phenotypes [21]. Because in such a system very little donor DNA is retained in the recipient cell and non-linked genes are not normally co-transferred, we again concluded that both the CH^R and MDR phenotypes are the result of a single gene mutation.

I have gone into some detail with respect to the ''somatic cell'' genetic investigation of the CH^R phenotype, because it led us to the fundamental premise that a single genetic alteration was the most likely explanation for the complex MDR phenotype. When P-gp was later discovered, and its level of expression varied with the drug resistance phenotype in the ''genetically defined'' cell lines (independent isolated clones, revertants, DNA transfectants, etc.), we were fully convinced that it must play a critical and likely causative role. In many respects, these well-characterized lines allowed us to define P-gp functionally before its primary sequence was known.

We were fortunate to have a strong membrane biology community at the University of Toronto. When Rudy Juliano, a young faculty member in Biophysics in Aser Rothstein's group at the Research Institute, Hospital for Sick Children, heard about our CH^R permeability ''membrane'' mutant cells, he proposed to investigate the cells with the recently developed labeling technique to label cell-surface carbohydrates using galactose oxidase-[^{3}H]-borohydride [22,23]. That was a defining moment – when we saw a very prominent 165 000 MW peak, significantly larger than any other peak in the gel. This peak was observed in CH^R mutants, not in wild-type cells, and it was greatly reduced in revertant cells. Moreover, it was present in an actinomycin D Syrian hamster line. We called it P-glycoprotein (P-gp) because of its association with colchicine permeability.

In those early cell surface-labeling experiments, a dramatic increase in the actual amount of amino acid labeled protein could not be demonstrated. Although we did not know the reasons for this, we speculated that the prominent surface labeling in mutant cells could be due to some general conformation change on the cell plasma membrane exposing more carbohydrates rather than the result of increased synthesis, consistent with the notion of a pleiotropic alteration. Of interest also was the fact that Rudy used the Fairbanks gel system to detect P-gp where the sample was dissolved in SDS and urea [24], a system favored by membrane biochemists at that time. We did not realize until much later how important the choice of the gel separation system was for detecting P-gp. Lee Greenberger and Susan Horwitz took the initiative to determine if the ''P-glycoproteins'' they detected in their mouse cell line were different from the CHO cell P-gp. In a collaborative study, Lee showed that P-gp was detected with a much lower efficiency when prepared for separation in the more popular Laemmli gel system (SDS plus heating) compared with the Fairbanks system [25]. Apparently, the heating step caused the amount of P-gp detected to be greatly reduced in the Laemmli system, while this was not the case in the Fairbanks system. This fact may have prevented many from detecting the presence of P-gp in their system.

Jack Riordan at the Hospital for Sick Children approached us to purify P-gp using his membrane biochemistry expertise. He isolated plasma membrane vesicles (PMVs) from the series of mutants and revertants and showed that PMVs from the highly resistant CH^R C5 cells contained a prominent Coomassie blue stained band at about 170 000 molecular weight resolved by Fairbanks gel electrophoresis. This band was less prominent in the less resistant CH^R C4 cells and not present or barely detectable in the wild-type or revertant cells [26]. Extraction of the PMVs with various mild agents showed that P-gp could not be extracted with other membrane peripheral proteins so it was likely an integral membrane protein. It could be solubilized with non-ionic detergents and greatly enriched using a series of lectin affinity columns to a level of purity such that it was easily the major band in the SDS gel with some contaminating minor bands. This was another defining moment, as it showed for the first time clear biochemical evidence that P-gp is localized in the plasma membrane, that it is present in significant amounts (estimated to be 3–4% of total plasma membrane protein) in a highly colchicine-resistant line and that the amount of this protein in the plasma membrane correlated with the level of colchicine resistance. Moreover, it raised the possibility that it might be possible to use the isolated PMVs for in vitro drug uptake studies and that the protein could be purified for reconstitution studies and used to raise antisera. Norbert Kartner was involved in that work as a research assistant in Jack's lab and he caught the P-gp ''bug''. He applied to work as a graduate student in my lab, thereby cementing the collaboration between our two labs.

Norbert decided to generate rabbit antisera directed against the cell surface components of the multidrug-resistant human CEM/VLB_{100} cell line selected for resistance to vinblastine

by Bill Beck [27] or to the highly resistant CH^R C5 CHO cell line to improve the sensitivity and specificity of detecting P-gp [28]. To our delight, the antisera detected P-gp in different MDR cell lines from different species isolated with different drugs. He used a Western blot analysis to show all these lines had one membrane component in common, a ~170 000 MW component that increased with the level of drug resistance – P-gp. In fact, when the antiserum raised against the human CEM/VLB_{100} cell line was used against CHO cell membranes of different levels of drug resistance, the only component detected was P-gp. The antiserum did not detect any other membrane component. This was another defining moment, as we could now quantify with some degree of accuracy the amount of P-gp expressed to correlate with other phenomena. In this context, the Western blot analysis of the L-cells transfected with genomic DNA from CH^R C5 cells [21] expressed P-gp; moreover, the level of expression correlated with the degree of colchicine resistance and with the number of double minute chromosomes (DMs) [29]. Since DMs are associated with gene amplification, we predicted that the P-gp gene was amplified in the transfected cells and possibly in other selected MDR cell lines [28,29]. We speculated also that it should be possible to clone the P-gp gene from isolated DMs, as Caizzi and Bostock [30] had done in cloning amplified DNA sequences in methotrexate-resistant cells.

Norbert Kartner further developed the theme that P-gp was similar in different MDR lines by generating a series of monoclonal antibodies using a dot blot assay on nitrocellulose to screen for hybridoma clones producing antibodies able to recognize membrane proteins from different species of mammalian resistant cell lines but not sensitive ones. He isolated three classes of monoclonal antibodies [31], and their binding epitopes were later defined by Elias Georges [32]. One of them, C219, appeared to recognize a highly conserved epitope, the c-terminal half of P-gp, localized in the cytoplasmic domain. This monoclonal antibody was used by Jack Riordan to screen a λ gt11 cDNA expression library from a derivative of CH^R C5 cells (CH^R B30) that Norbert had "bumped up" so that it could grow in 30 μg/ml of colchicine. A 600 bp cDNA probe, λ CHP1, was isolated. The lacZ fusion product of λ CHP1, which expresses a C-terminal fragment of P-gp, is recognized by monoclonal antibodies that identify three different epitopes of P-gp. The Southern blot analysis of different CH^R lines revealed that the degree of drug resistance correlated with the amplification of λ CHP1 homologous sequences and that P-gp is encoded by a multigene family clustered in one amplicon [33]. Soon after these speculations, Alexander Van der Bliek in Piet Borst's group used pulse field gel electrophoresis to characterize this amplicon showing that it contains at least 5 genes [34].

With the availability of a cDNA probe for P-glycoprotein, we cloned and sequenced the 3′ half of the P-gp cDNA from the wild-type CHO cell line. The close similarity of this sequence with the bacterial hemolysin B transport protein stunned us [35] and it caused us to formulate, by analogy to hemolysin B, a functional model of P-gp as a multidrug efflux pump. This led us and others to renew our effort to purify, reconstitute, and to investigate the properties of this remarkable protein (see Refs. [12,13,36–38]). It seems clear that P-gp can act alone as a multidrug transporter. What is not clear is whether this transport function of P-gp is sufficient to account for other pleiotropic aspects of the MDR phenotype. There is more to be learned.

3. Molecular biology and mechanism of multidrug resistance (1980–1990): Michael Gottesman and colleagues

When I joined NCI in 1976 as a senior investigator, my first goal was to establish somatic cell genetic systems for the study of processes essential for cancer growth. My first post-doctoral fellow, Fernando (Buz) Cabral was an expert in 2D gel analysis, and I was experienced in developing drug-resistant bacterial mutants. We reasoned that a robust cultured cell system could be selected in anti-microtubule drugs frequently used to treat cancer, and we would be able to use 2D gels to determine what targets were mutated in the resistant cells. Owing to the pioneering work of Theodore Puck (University of Colorado) in developing Chinese Hamster Ovary (CHO) cells as such a culture system, and thanks to the generosity of Louis Siminovitch (University of Toronto), who had developed subclones of CHO cells that grew easily in suspension and had also demonstrated the power of this system to facilitate isolation of somatic cell mutants, we decided to begin with CHO cells. Our first work resulted in the isolation of β-tubulin mutants [39] that were temperature-sensitive for growth [40] and we used the mutants to study spindle formation and how anti-microtubule drugs work in the treatment of cancer.

In 1981, Ira Pastan (my lab chief in the Laboratory of Molecular Biology, Division of Cancer Biology and Diagnosis, NCI) and I were approached by Bruce Chabner, Director of the NCI's Division of Cancer Therapy, who pointed out that the real problem in treating cancer was not single agent resistance, but MDR. I used to refer to this observation as the "gospel of Bruce Chabner" – this appears in virtually every paper on this subject in the opening words "Multidrug resistance is a major impediment to the successful treatment of cancer". Bruce asked Ira and I to consider working on MDR and I became the leader of a small group dedicated to determining the molecular basis of MDR in cancer. Alan Rabson, our own Division Director, was a strong supporter of this effort, and we were given additional resources to attack this problem. We wrote a strategic plan that emphasized development of reagents suitable for use with human samples so that we could apply our results immediately to human cancers. Our initial approach was to develop MDR cultured human cancer cells, use these to clone human MDR genes, and develop diagnostic anti-human MDR gene product antibodies. By this time, the MDR phenotype of cultured cells had been described, and Rudy Juliano and Victor Ling [22] had already done their pioneering work on expression of P-gp in MDR CHO lines, but it was by no means certain that P-gp was the protein responsible for MDR, or that if it did contribute to MDR, that it was sufficient to confer the complete MDR phenotype.

Shin-ichi Akiyama, a fellow in the lab, began by screening human cultured cancer cells for two properties: high efficiency of cloning and sensitivity to common MDR drugs such as colchicine, vinblastine and doxorubicin. Among a half dozen or so cell lines we tested, one stood out – a line called KB which was reported to be a human nasopharyngeal carcinoma. After we began working with this line we discovered that it was actually a subclone of HeLa, a cervical adenocarcinoma cell line that had contaminated and overgrown quite a few putative human cancer cell lines and had been acquired by ATCC and distributed as KB. To this day, HeLa (KB), owing to its robust

growth properties and drug sensitivity, has proved to be a very useful human cancer cell line for studying development of drug resistance.

Akiyama isolated many single-step clones of colchicine-resistant KB cells [41]. We chose one such clone (KB-8) because it was cross-resistant to the drugs of interest and then proceeded to isolate other clones in multiple steps with increasing levels of MDR, working only with those clones with similar patterns of MDR in the hope of amplifying the gene or genes responsible for this resistance pattern. This strategy was based directly on the work of Robert Schimke (Stanford University) who had shown that gene amplification is a common mechanism of drug resistance in cultured cells, and we hoped that amplifying a gene would facilitate its eventual cloning. The KB cell line series (KB-8, KB-8-5, KB-8-5-11 and subsequent, more resistant populations such as KB-C1) have subsequently been shown to overexpress only MDR1 among all 48 known ABC transporters, supporting our original strategy for selection. KB-8-5 was subsequently found to have a level of MDR1 expression similar to many human cancers [42]. Consequently, these cells are still widely used as standards for human P-gp expression by researchers and pharmaceutical firms throughout the world. Surprisingly, we did not see significant P-gp expression in any of these cell lines as analyzed by Coomassie Blue and silver staining of one-dimensional and two-dimensional gels (no antibodies were as yet available to P-gp); this result turned out to be due to technical problems associated with detection of P-gp on Laemmli gels (see section above). Once we had learned how to isolate MDR cell lines from KB cells, we repeated these selections in the presence of vinblastine (KB-V series) and doxorubicin (Adriamycin)(KB-A series). Having several different selected cell lines, otherwise isogenic, turned out to be a key element in the cloning of the *MDR*1 gene.

While we were puzzling about the best way to isolate *MDR*1 genes overexpressed in our cell lines, I attended a Gordon Conference in New Hampshire and at a poster session met Igor Roninson, who was finishing his graduate studies with Alex Varshavsky and had developed a generalizable technique for cloning amplified genes from cultured cells. The technique involved in-gel denaturation and renaturation of DNA that had been treated with a restriction enzyme. The renaturation conditions were set so that only highly amplified genes would become double-stranded in the gel; single-stranded DNA could then be digested with a single-strand specific nuclease. The beauty of this technique was that with several different MDR cell lines we could look for amplified human genes that were found in common and we found them in the KB cell lines [43]. Roninson, working with David Housman and Varshavsky, had already demonstrated the utility of this technique in MDR Chinese hamster cells [44]. Two kinds of human MDR genes were found that we called *MDR*1 and *MDR*2 [43], but an mRNA of 4.5 kb was only initially identified for the *MDR*1 gene [45]. This was the beginning of a long and fruitful collaboration with Igor Roninson, an extraordinarily creative and consistently thoughtful colleague in the field of MDR. In the mouse and hamster, MDR genes were also being cloned at around the same time in the laboratories of Victor Ling, Peter Melera (University of Maryland), Piet Borst (Netherlands Cancer Center), and Philippe Gros (McGill University). There are two mdr1-like genes in rodents and these came to be called *mdr*1 and *mdr*3 in the mouse by Philippe Gros and *pgp*-1 and *pgp*-2 in the hamster by Victor Ling's group. Susan Horwitz (Albert Einstein College of Medicine) suggested the compromise that the rodent mdr1-like genes be called mdr1a and mdr1b, but there remains a difference of opinion about whether *MDR*2 or *MDR*3 should be the name of the second human MDR gene. When the human gene mapping organization (HUGO) met to consider nomenclature of ABC genes, MDR genes were called ABCB1 and ABCB4, a decision that has certainly confused matters further.

Having a full-length *MDR*1 gene in hand allowed us to do many critical experiments to answer the burning questions of the day regarding MDR. The first major question was whether or not *MDR*1 encoded P-gp. We collaborated with the Ling lab to demonstrate that the protein product of the *MDR*1 gene was indeed P-gp in a little referenced, but critical paper published in the BBRC [46]. As can be well-imagined, there were high level discussions about authorship of this short manuscript, and we settled on having NCI fellows including Kazu Ueda, a brilliant post doc in our lab who had created the first full-length expressing clone of human P-gp, occupy the first author slots, and Victor and Jack Riordan, a longtime collaborator of the Ling laboratory, as the final authors. This manuscript brought some important order to the MDR field by marrying the molecular biology with the biochemistry and demonstrating that it was okay for competitors to collaborate.

The second major question was whether MDR1 (P-gp) was able to confer the entire phenotype of multidrug resistance. As noted, Kazu Ueda had created a full-length clone in a retroviral expression system [47] and in short order was able to show that individual transfectant cell lines were fully multidrug-resistant. Our paper appeared a few months after a similar demonstration by the Gros lab using mouse mdr1 [48], which was a bit of a disappointment, since we thought this was probably our most important contribution to date. Philippe Gros, starting his career as an Assistant Professor in McGill, was thinking about MDR in much the same way that we were and continued for several years to produce interesting and important papers focusing on mouse *mdr*1a and *mdr*1b genes.

Given that we had undertaken our studies on MDR with the intent of applying them to human cancers, we moved quickly to determine in what tissues MDR1 was normally expressed and whether it was expressed in human cancers at levels that could lead to MDR. Another collaboration, with Takashi Tsuruo of the Tokyo Chemotherapy Center, proved to be critical here. Tsuruo's lab had been a pioneer in showing that verapamil could reverse MDR [49] (verapamil was later shown to be a substrate and competitive inhibitor of P-gp), and he had also produced a monoclonal antibody, MRK16, that specifically detected human P-gp [50]. Unlike the anti-hamster P-gp monoclonal antibody, C219, which was the only other antibody available at the time but was not absolutely specific for P-gp, MRK16 did not detect any other proteins and seemed ideal for our purposes. Tsuruo was very generous with the antibody and coauthored with us the first paper describing the pattern of human P-gp expression in the apical portion of intestinal, liver, and kidney epithelia [51,52]. None of this immunohistochemistry would have been possible without the leadership of Mark Willingham, a longtime collaborator of Ira Pastan's in the Laboratory of Molecular Biology, NCI, who was a wizard with microscopes and imaging. This work also illustrated the power of tapping the multidisciplinary team

that Ira Pastan had assembled in the Laboratory of Molecular Biology in NCI.

The localization result was confirmed by another outstanding fellow in the lab, Tito Fojo, using molecular analysis with cDNA probes to detect mRNA [53]. Tito was a clinical fellow in the NCI who had chosen to do research with Ira Pastan and me, and proved to be an extremely productive and innovative MDR investigator who focused on clinical relevance of our experiments. Another such fellow, Lori Goldstein, was assigned the task of determining whether MDR1 was expressed in human cancers. She collected close to 1000 human cancers, developed tools for isolation of non-degraded mRNA from these tumor samples, and learned how to quantitate mRNA on a slot blot. The success of these experiments [42] showed that MDR1 was expressed at levels judged to be sufficient to confer MDR in many epithelial cancers derived from tissues that were known to express MDR1 (colon, liver, kidney), was found in hematopoeitic cancers (AML, ALL. lymphoma) and solid tumors (breast, ovary) that had relapsed after chemotherapy with P-gp-affected agents, and also appeared occasionally in cancer simply as a result of the transformation process. One important assumption made throughout these studies was that the expression of MDR1 mRNA would correlate with functional P-gp; subsequent studies have shown this to be correct with few, if any, exceptions. However, we have come to believe strongly that localization of P-gp should be demonstrated by two techniques before the result can be taken seriously; one of these should be molecular, while the second could use an antibody to confirm that the protein is on the plasma membrane, where it can be functional.

Based on these expression studies and others done in our lab and elsewhere, Ira Pastan and I speculated that P-gp had evolved as a general protection mechanism to keep toxic xenobiotics out of the body (intestinal barrier), to excrete such xenobiotics from the kidney and liver if they were absorbed, and to protect vulnerable tissues from circulating toxins (blood–brain, blood–testis/ovary, and placental barriers). This hypothesis was first enunciated by Thiebaut et al. [51], and in a review for the New England Journal of Medicine that we wrote [54] and has become a central part of the P-gp dogma. Pharmaceutical and academic interest in the effect of polymorphisms of P-gp on the pharmacokinetics of many common drugs derives directly from this hypothesis. Later, we produced transgenic mice in which MDR1 was overexpressed in bone marrow, and showed that this expression did, indeed, protect mouse tissues from toxic levels of xenobiotics [55].

The studies on expression of P-gp in tumors and normal tissues led naturally to questions about how this expression was regulated. Kazu Ueda was particularly interested in this issue and isolated the downstream *MDR*1 promoter [56]. This promoter has since been analyzed in much more detail by Kathleen Scotto (Fox Chase Cancer Center) and her colleagues, among others [57], but one interesting discovery at the time was that in some of our KB-MDR cell lines the majority of transcription came from a distant upstream promoter [58]. In more recent independent work, Tito Fojo and colleagues have shown that rearrangements involving this upstream promoter frequently occur in drug-resistant cancers, providing the most convincing direct evidence to date that selection for MDR1 expression occurs in vivo in human cancers [59].

Although the molecular biology of the *MDR*1 locus was being pursued in the laboratories of Piet Borst, Phillipe Gros, and Igor Roninson by 1986, not much was known about how a single protein could confer resistance to so many different drugs. The original designation of P-gp was based on the assumption that this protein somehow blocked uptake of many different anti-cancer drugs (P stood for permeability in the original Ling and Juliano work), but it was clear that the major mechanism of resistance was increased drug efflux [7,60]. This mechanism suggested that P-gp could bind drugs directly, and we set out to demonstrate this. The initial studies done by Marilyn Cornwell in our lab showed specific binding of vinblastine to membrane vesicles containing P-gp, but it became clear that we needed a photoaffinity label to directly demonstrate binding of drugs to the putative transporter. Once again, a collaboration with two NCI investigators, Ron Felsted and Ahmed Safa, proved crucial. Safa had appreciated the need to develop photoaffinity analogs of vinblastine for use in studying binding to tubulin, its major intracellular target, and worked with us to adapt this system for studies of P-gp [61]. In similar fashion, Cornwell demonstrated binding of ATP to P-gp [62] and later, Ursula Germann showed ATPase activity in membranes containing P-gp [63]. The P-gp baculovirus that Germann created in our lab turned out to be an extremely useful reagent for Balazs Sarkadi (National Medical Center, Hungary), an expert on ATPases, who has done some elegant work on ATPases associated with P-gp and several other ABC transporters [64]. With drug binding and ATPase activity demonstrated for P-gp, it seemed increasingly likely that it was the energy-dependent transporter responsible directly for MDR.

One missing piece of data was whether P-gp could act alone to transport drugs. Hypotheses about P-gp working indirectly by affecting membrane potential or proton transport were proposed in the literature, and although these seemed unlikely based on the amount of drug being transported and the lack of pH or potential gradients in many MDR cell lines, we sought direct evidence that pure P-gp could transport drugs in isolated membrane vesicles. Another talented Japanese fellow, Masuro Horio, trained in transport physiology, was able to show that inside-out membrane vesicles containing P-gp could transport drugs into the vesicle [65], but it required the biochemical skill of another colleague, Suresh Ambudkar, then at Johns Hopkins but eventually to become a senior investigator and highly valued colleague in the Laboratory of Cell Biology, NCI, to reconstitute purified P-gp into vesicles and demonstrate transport and ATPase activity [66]. Similar studies were done with highly purified P-gp in Ling's lab by Shapiro ([36,37]; see above). Horio, in a collaboration with another NIH colleague and transport expert Joe Handler, also showed that P-gp could catalyze transepithelial transport in both transfected [67] and naturally occurring cell lines [68]. This assay, using dog, pig, or human kidney cells, is now a standard test for transepithelial transport by P-gp and other ABC transporters whose expression is polarized.

How did P-gp recognize so many different substrates? Although we and others have done detailed mutational analyses to identify important residues that affect substrate specificity and it was clear that even a single point mutation could change substrate specificity [69], everyone was having trouble understanding the apparent promiscuity of the transporter. Once again, a collaborator came to our rescue. Yossi Raviv,

an Israeli scientist on sabbatical in the laboratory of Robert Blumenthal, another NCI senior investigator, was looking for systems to study with a non-specific, hydrophobic affinity probe (INA or iodonaphthalene azide). This probe, after UV activation, labeled most proteins with transmembrane segments, and, indeed, in control experiments, P-gp was heavily labeled by ^{131}I-INA. Raviv worked out a way to indirectly label proteins that interact with chromophores; we showed that daunorubicin, when photoactivated, could transfer energy to INA which would specifically label P-gp [15]. Since INA is only present in hydrophobic regions of membranes, the interaction of daunorubicin with P-gp must have been within the plasma membrane. This observation resulted in two models that have influenced mechanistic thinking about P-gp and several other ABC transporters: the hydrophobic vacuum cleaner model [15] and the flippase model [14]. In the hydrophobic vacuum cleaner model, drugs are detected and extruded from the lipid bilayer into an aqueous environment; thus, P-gp is effectively a phase separator that takes drugs from a hydrophobic environment and places them in an aqueous environment. The flippase model, that derived directly from conversations with Robert Blumenthal, an NCI biophysicist who pointed out to me the resemblance of the drug transport process to lipid flipping, proposes that drugs from the inner leaflet are flipped to the outer leaflet by P-gp, where they can diffuse into the aqueous environment around the cell, or bind to proteins such as albumin, present in medium. In practice, there is no easy way to distinguish these two models, since drugs are in very rapid equilibrium between the extracellular environment and the outer leaflet of the plasma membrane. Experimental evidence from many different systems has since confirmed that one of these two models is likely correct.

As the 1980s came to a close, we began to think about ways to exploit our knowledge about P-gp. We were told by our colleagues who were oncologists that cancers seemed able to easily develop MDR, but normal tissues never became resistant to the toxic effects of chemotherapy. So we set about to use P-gp vectors to protect normal tissues against cytotoxic MDR substrates, and to use these vectors to enable selection of otherwise non-selectable genes [55,70,71]. Detailed clinical studies on the role of P-gp in individual human cancers were obviously essential, and depending on the outcome of these studies, effort needed to be devoted to develop agents that were not P-gp substrates (evasion of P-gp), other drugs that were inhibitors of P-gp (engagement of P-gp), or drugs that specifically kill P-gp expressing cells (exploiting the Achilles heel of P-gp) [72]. These concepts were reviewed recently in Szakacs et al. [73].

4. Concluding remarks

It has been 30 years since the discovery of P-gp. We welcome this opportunity to reflect on some of the early work and especially on those defining moments that caused us to change our perspective. This is a story of convergence, of different research approaches and concepts. The Ling lab used somatic cell genetics to investigate the properties of colchicine resistance in Chinese hamster ovary cells. This resulted in linking P-gp to MDR. The Gottesman lab used molecular biology to clone amplified DNA sequences associated with the MDR phenotype in cell lines. Both approaches converged and the P-gp gene and the MDR gene were subsequently shown to be the same [46]. This has led to the current acceptance of P-gp as an efflux pump of broad specificity providing an explanation of the molecular basis for multidrug resistance. At the same time, the lack of expression of P-gp in some multidrug-resistant cell lines, notably lung cancer cells, led to the search for and discovery of the second ABC transporter that confers multidrug resistance, MRP1 ([74]; also see chapter by Deeley and Cole in this volume). This is a story of how science progresses, by collaboration of creative individuals and by adoption of new technologies as they emerge to answer questions of immediate interest and importance.

Acknowledgement: We are grateful to George Leiman for his outstanding editorial assistance.

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