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Unidirectional fluxes of rhodamine 123 in multidrug-resistant cells: Evidence against direct drug extrusion from the plasma membrane

(P-glycoprotein/multidrug resistance/doxorubicin/vinblastine)

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ABSTRACT **P-glycoprotein (Pgp), a plasma membrane protein overexpressed in multidrug-resistant tumor cells, is an ATPase thought to actively export cytotoxic drugs. It has been proposed that Pgp transports drugs directly from the lipid bilayer to the external medium ("vacuum cleaner" hypothesis). A possible mechanism for this model is that the Pgp is a flippase—i.e., it catalyzes the translocation of hydrophobic substrates from the inner to the outer leaflet of the cell membrane. Two immediate predictions of the vacuum cleaner and flippase hypotheses are that the apparent unidirectional influx of substrate should be less in Pgp-expressing than in Pgp-lacking cells and that this difference should be abolished by inhibition of the Pgp. We used Chinese hamster fibroblasts with different levels of Pgp expression to measure true unidirectional fluxes of rhodamine 123 (R123), a Pgp-transported fluorescent dye that accumulates in mitochondria (hence, its cytosolic concentration remains low at short times after external addition). The unidirectional efflux of R123 was proportional to the level of Pgp expression and was reduced by Pgp inhibitors. The unidirectional influx of R123 was the same in sensitive and resistant cells—i.e., independent of the level of Pgp expression and insensitive to inhibitors of R123 efflux. From these results, we rule out the vacuum cleaner and flippase hypotheses and conclude that Pgp extracts the actively transported substrates from the cytosol and not from the plasma membrane.**

Multidrug resistance (MDR) caused by overexpression of P-glycoprotein (Pgp) is attributed to enhanced drug efflux that results in reduced intracellular steady-state levels of cytotoxic drugs thought to enter the cell by solubility–diffusion across the phospholipid moiety of the plasma membrane (1, 2). It is thought that Pgp is both a drug efflux pump and a Cl^- channel activated by cell swelling (3–7). Recently, it has been suggested that its pump function may involve substrate transport from the lipid bilayer itself to the external solution ("vacuum cleaner" hypothesis; see ref. 8). Within this hypothesis, Pgp could be a flippase—i.e., it could catalyze the translocation of hydrophobic substrates inserted in the cell membrane from the inner to the outer leaflet (9). According to either model, drug "efflux" would occur, at least in part, before the drug molecules gain access to the cell interior. The idea that drugs might be extruded from the membrane itself is principally based on (*i*) the hydrophobicity of many Pgp substrates, (*ii*) the demonstration by fluorescence energy transfer that in Pgp-expressing cells doxorubicin and rhodamine 123 (R123) are virtually completely associated to Pgp, (*iii*) the broad substrate specificity of the Pgp (see refs. 9–11), and (*iv*) the contention that unidirectional influx is reduced in MDR cells (12, 13). Two predictions arise from these hypotheses. First, the apparent unidirectional influx of Pgp substrates should be reduced in Pgp-expressing cells because part of the substrate partitioning into the plasma membrane would be transported outward by Pgp and hence not reach the cell interior. Second, inhibition of Pgp function, by substrate competition, block, or ATP depletion, should cause not only a decrease in efflux but also an increase in influx.

To test whether in MDR cells drugs are directly extruded from the membrane, we used R123, a fluorescent substrate of Pgp. We chose R123 for two reasons. (*i*) It is transported by Pgp at a rate that permits quantitative analysis of the efflux with sufficient time resolution to measure transient changes in external solution during an efflux determination (14). (*ii*) Because R123 is accumulated in mitochondria (15), during the influx experiments the cytoplasmic concentration is lower than that of evenly distributed dyes. The second feature makes R123 a good probe to measure "true" unidirectional influx—i.e., influx uncontaminated by efflux.

MATERIALS AND METHODS

Experiments were performed on subconfluent monolayers of wild-type (V79) and progressively MDR Chinese hamster lung fibroblasts (77A, LZ-3, and LZ-8) (16–18) grown as described (14). Pgp is expressed in the plasma membrane of all of these cell lines; the levels are reported to be 20-fold greater in LZ-8 than in V79 cells (18).

In most experiments, we used a HCO_3^-/CO_2-buffered solution with the following composition: 115 mM NaCl, 25 mM $NaHCO_3$, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 1.5 mM sodium phosphate, and 7.8 mM glucose, equilibrated with 95% O_2/5% CO_2 (pH 7.42–7.43). The solutions buffered with Hepes contained 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 7.8 mM glucose, and 5 mM Hepes, titrated with NaOH to pH 7.42–7.43.

Steady-State Intracellular Drug Levels. Cells were exposed to 15 μM R123 for 60 min at 37°C, washed for 30 s with an ice-cold solution containing 100 μM vinblastine to inhibit R123 efflux (14), placed in a cuvette, and lysed with distilled water. The R123 content was determined by fluorometry as described (14).

Steady-state vinblastine levels were determined as described (19). Cells in Hepes-buffered solution were loaded at room temperature with [^{3}H]vinblastine (55 nM; 0.5 μCi/ml; 1 Ci = 37 GBq) for 2 h, washed in ice-cold phosphate-buffered solution without [^{3}H]vinblastine, and then treated with trypsin. Radioactivity was quantitated by scintillation counting and cell number was determined from a nuclei count (20).

Unidirectional Efflux of R123. The decay of intracellular R123 fluorescence (F_{R123}) was measured from 100–150 cells plated on round coverslips. After R123 loading (see above) the coverslips were mounted in a Leiden microincubator (Medical Systems, Greenvale, NY), on the stage of an

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Abbreviations: MDR, multidrug resistance or multidrug resistant; Pgp, P-glycoprotein; R123, rhodamine 123; F_{R123}, R123 fluorescence; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone.

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inverted microscope (Nikon Diaphot), and superfused at 37°C with R123-free solutions at a rate of ≈20 ml/min (the $t_{1/2}$ of the extracellular washout was 1–2 s). Except at very high loading levels—i.e., in cells loaded in the presence of inhibitors of R123 efflux—F_{R123} decays with a time course that is well fit by a single exponential. At the highest F_{R123} (in the presence of efflux inhibitors), there is significant R123 quenching in mitochondria, as evidenced by the increase in fluorescence upon collapsing the mitochondrial inner membrane voltage with the protonophore carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (21).§ Rate constants for F_{R123} decay (k) were estimated from fits of Eq. 1 to the data:

$$F_{R123} = F_0 + F_{R123}(0)e^{-kt}, \quad [1]$$

where F_0 is the background fluorescence and $F_{R123}(0)$ is F_{R123} at $t = 0$.

Unidirectional Influx of R123. The unidirectional influx of R123 was determined from efflux data by extrapolation to $t = 0$. Cells were exposed to control solution containing 15 μM R123 for periods ranging from 6 s to 60 min, the fluorophore was removed, and the decrease in F_{R123} was recorded. $F_{R123}(0)$ was determined (for each exposure time) extrapolating the fit of Eq. 1 to $t = 0$, hence yielding an estimate of the intracellular R123 amount at the end of the loading period. $F_{R123}(0)$ values were normalized to the steady-state R123 levels. This analysis assumes no quenching, which is supported by data presented in *Results and Discussion*. The k values for unidirectional efflux were independent of the duration of the loading period (e.g., 0.297 ± 0.044 and 0.288 ± 0.030 min^{-1} in V79 cells exposed to R123 for 6 and 60 s, respectively). Up to five R123 influx determinations were performed on the same cells.

R123, verapamil, and FCCP were purchased from Sigma; vinblastine sulfate was from Lyphomed (Deerfield, IL); and [G-^{3}H]vinblastine sulfate was from Moravek Biochemicals (Brea, CA).

Data shown are means ± SEM. Statistical comparisons were done by Student's t test for paired or unpaired data as appropriate. Differences were considered significant when P was <0.05.

RESULTS AND DISCUSSION

V79 and LZ-8 cells have a low and a high degree of MDR, respectively (14, 18, 24). Accordingly, the IC_{50} values (drug concentrations causing 50% inhibition of colony formation) were greater in LZ-8 than in V79 cells. IC_{50} ratios (LZ-8/V79) were 2100 and 90 for doxorubicin (Adriamycin) and vinblastine, respectively. Verapamil (25 μM) alone had no effect by itself on cell survival but increased the sensitivity to doxorubicin and vinblastine. IC_{50} values increased 10- and 32-fold for doxorubicin and vinblastine in V79 cells and 650- and 250-fold for the same drugs in LZ-8 cells. These effects of verapamil support the previous conclusion (18) that Pgp mediates the MDR of both cell lines.

Fig. 1*A* shows steady-state R123 intracellular levels in V79 and LZ-8 cells.¶ The levels are significantly lower in LZ-8 compared to V79 cells. Verapamil increased R123 levels in both cell lines. Vinblastine, a nonfluorescent substrate of Pgp, also increased the steady-state R123 levels. Fig. 1*B* shows that the steady-state intracellular level of vinblastine is lower in LZ-8 than in V79 cells and that verapamil increased the steady-state intracellular accumulation of vinblastine in both cell lines. These results are in agreement with the demonstration of functional Pgp expression in both cell lines, but at a higher level in LZ-8 cells (18).

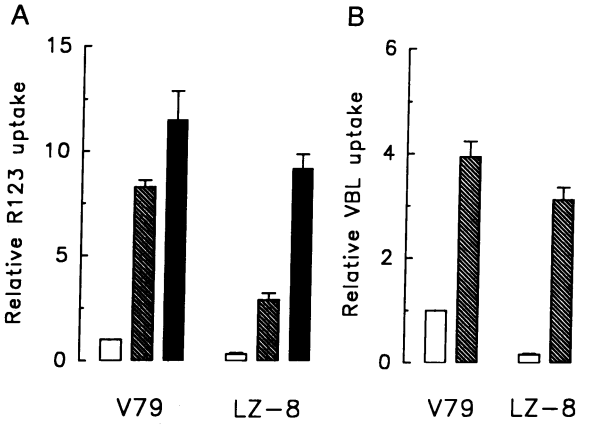

FIG. 1. (*A*) Steady-state intracellular levels of R123 in V79 and LZ-8 cells, under control conditions (open bars), with 100 μM verapamil (hatched bars), or 10 or 100 μM vinblastine (solid bars) in V79 and LZ-8 cells, respectively. In V79 cells, 10 μM vinblastine had maximal effect; hence, in most experiments we used this concentration. (*B*) Steady-state intracellular levels of vinblastine (VBL) in V79 and LZ-8 cells under control conditions (open bars) and with 100 μM verapamil (hatched bars). Data shown are means ± SEM of 6–17 experiments, normalized to the uptake in V79 cells in the absence of modulators. Steady-state R123 and vinblastine levels in V79 cells were 71.7 ± 7.5 (n = 17) and 6.0 ± 0.5 pmol per 10^6 cells (n = 6), respectively.

Records of the decay in F_{R123} upon removal of extracellular R123 are shown in Fig. 2*A*. The decrease follows a single-exponential function of time in both V79 and LZ-8 cells (best fits are superimposed on fluorescence data). As expected from the drug-resistance studies above, and previous estimates of plasma membrane Pgp levels (18), the R123 efflux was faster in LZ-8 than in V79 cells. Fig. 2*C* summarizes rate constants of R123 efflux in cell lines expressing progressively higher degrees of MDR. The amounts of Pgp expressed at the plasma membrane, relative to those of the wild-type V79 cells, were 2-, 10-, and 13-fold in 77A, LZ-3, and LZ-8 cells, respectively (18). The rate constants correlate with Pgp expression. However, in highly resistant cells increases in resistance to doxorubicin occur without changes in R123 efflux—e.g., LZ-8 cells are 4-fold more resistant to doxorubicin than LZ-3 cells, although the plasma membrane Pgp expression is only 30% greater. These observations are in agreement with previous reports (18, 25, 26) indicating that Pgp expression and MDR are linearly related only at low levels of Pgp expression.

Fig. 3*A* shows inhibition of R123 efflux by transient exposure to vinblastine in V79 cells. Verapamil had similar effects (data not shown). The inhibitory effects started within 10 s of exposure and were fully and rapidly reversible. This directly demonstrates inhibition of Pgp-mediated unidirectional efflux. Fig. 3*B* summarizes the rate constants of R123 efflux in the absence and presence of verapamil or vinblastine. Both agents slowed the decay in F_{R123} in cells exposed to FCCP (data not shown), indicating that their effects are exerted at the plasma membrane. These results demonstrate conclusively that the reduced intracellular accumulation of R123 in cells with high levels of Pgp is at least in part caused by an

§Mitochondrial R123 fluorescence quenching (22, 23) does occur in cells loaded in the presence of verapamil or vinblastine. Under these conditions, the F_{R123} can increase for a few minutes before decaying. However, upon loading without verapamil or vinblastine there is no F_{R123} quenching (assessed from the F_{R123} changes elicited by FCCP). Clearly, R123 efflux must be measured at low F_{R123}, a condition clearly met in V79 and LZ-8 cells loaded under control conditions.

¶R123 IC_{50} values of V79 and LZ-8 cells are >150 μM, indicating that both cell lines are highly resistant to the drug; the IC_{50} of a human breast cancer cell line with no detectable Pgp (MCF-7) (12) is ≈3 μM.

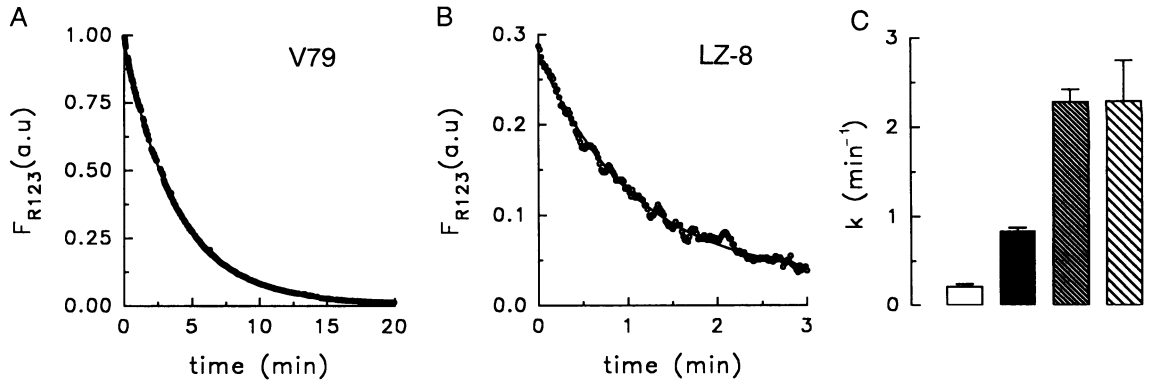

FIG. 2. (*A*) Typical record of intracellular F_{R123} decay in V79 cells. Cells were loaded as described. R123 was rapidly removed from the external medium and F_{R123} was measured as a function of time. (*B*) Typical record of F_{R123} decay in LZ-8 cells. In both cell lines, fluorescence decay is well fit by a single exponential. (*C*) R123 efflux rate constant in several progressively MDR Chinese hamster lung fibroblast cell lines (V79, open bar; 77A, solid bar; LZ-3, fine-hatched bar; LZ-8, coarse-hatched bar). Data are means ± SEM of 5–10 experiments.

increase in unidirectional efflux of the fluorophore. The inhibitory effects of verapamil and vinblastine support the conclusion that R123 efflux is mediated by Pgp.

Drug influx in cells expressing Pgp has been reported to be decreased relative to wild-type cells (12, 13). If the Pgp transports substrates from the lipid bilayer to the outside solution, then one would expect a reduced R123 unidirectional influx in Pgp-expressing cells. In addition, inhibitors of Pgp-mediated transport should cause an increase in unidirectional influx. Fig. 4*A* illustrates the relationship between the time of exposure to 15 μM R123 and the intracellular R123 fluorescence in V79 and LZ-8 cells. In V79 cells, a linear relationship was obtained only for loading periods of up to 15 s. In LZ-8 cells, the 6-s uptake did not differ from those at longer times, indicating that the linear uptake period is 6 s or less (see below). These results indicate that R123 permeability is high and hence that the unidirectional influx can be correctly estimated only from brief (<15 s) loading periods. Zero trans conditions hold for short loading times—i.e., the intracellular free R123 concentration is very low and hence the net flux approximates the unidirectional influx because the efflux is insignificant. Fig. 4*B* summarizes the influx data obtained in V79 and LZ-8 cells (after 6-s loading periods) under control conditions and in the presence of either verapamil or vinblastine. The influx rates of V79 and LZ-8 cells did not differ significantly. Furthermore, verapamil and vinblastine had no effects on the apparent R123 influx, although both drugs virtually abolished the efflux (Fig. 3). These results indicate that Pgp-mediated transport does not contribute to R123 influx and that the influx estimates are not significantly contaminated by Pgp-mediated R123 efflux. The absence of effects of verapamil and vinblastine on R123 influx and the almost complete inhibition of the efflux indicate that the dominant pathways for R123 influx and efflux are different and effectively independent. The observation of virtual abolishment of R123 efflux in the presence of the inhibitors may be explained by an extremely low free R123 concentration in the cytoplasm, which reduces the diffusive efflux, or to a chemical modification of R123 yielding a product with a lower diffusive permeability coefficient. A definitive conclusion will require further investigation.

We argue that the differences between the present results and the results of previous studies suggesting an effect of Pgp expression levels on drug influx (e.g., see refs. 12 and 13) can be ascribed to methodological differences. In some studies, estimates of drug influx in MDR cells were inadequate because of incorrect timing of the measurements—e.g., first measurement at 5–15 min (27, 28)—or because of poor choice of substrate—e.g., anthracyclines (12), which have clear disadvantages such as changes in fluorescence with lipid partition, binding to DNA and other factors (29), and rapid increase in cytoplasmic concentration during influx measurements. Finally, recent results showing an increase in apparent vinblastine influx by inhibitors of Pgp-mediated transport (13) are likely due to a decrease in efflux, and, as suggested by the authors, the estimated flux might not represent pure drug influx. The cytoplasmic concentration of vinblastine may increase faster than that of R123 under similar experimental conditions, because R123 is rapidly taken up by mitochondria (15). However, the possibility of real differences among results obtained with different drugs cannot be ruled out at present.

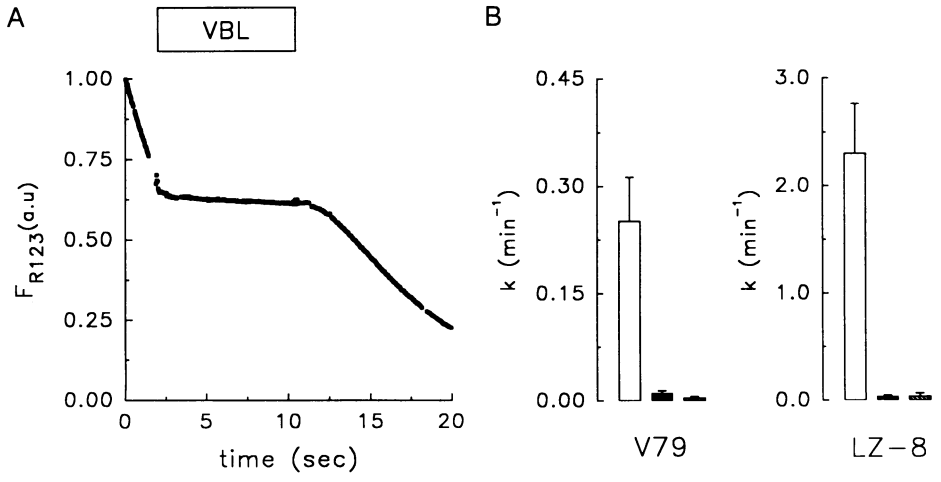

FIG. 3. (*A*) F_{R123} decay in V79 cells. Vinblastine (VBL; 10 μM) decreases reversibly the efflux of R123. (*B*) First-order rate constants for F_{R123} decay under control conditions (open bars), with 100 μM verapamil (solid bars), and with 10 or 100 μM vinblastine (hatched bars) in V79 and LZ-8 cells, respectively.

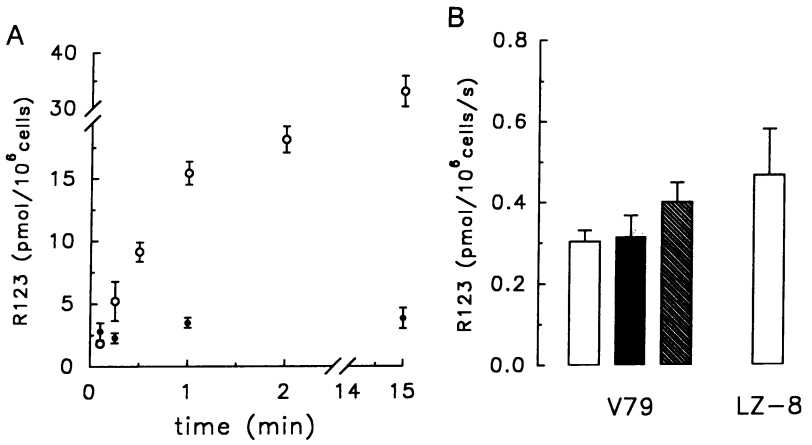

FIG. 4. (*A*) Intracellular R123 levels in V79 (open circles) and LZ-8 cells (solid circles) are plotted as a function of time of exposure to the fluorescent probe. (*B*) R123 influx, measured at 6 s of exposure, in the absence of inhibitors of R123 transport (open bars) and in the presence of 100 μM verapamil (solid bar) or 10 μM vinblastine (hatched bar). Data shown are means ± SEM of 5–8 experiments.

In summary, we have tested the hypothesis that Pgp transports substrates directly from the plasma membrane to the external solution (8–11). This hypothesis predicts reduced apparent influx in Pgp-expressing cells and restoration of influx levels upon Pgp inhibition. The results conclusively rule out the vacuum cleaner and flippase hypotheses. It seems more likely that the Pgp operates as an efflux pump, transporting hydrophobic substrates from the cytosol to the extracellular fluid. The apparent broad substrate specificity of this transporter awaits explanation.

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1. Beck, W. T. (1984) in *Molecular and Cellular Biology of Multidrug Resistance in Tumor Cells*, ed. Roninson, I. B. (Plenum, New York), pp. 21–228.
2. Roninson, I. B., Pastan, I. & Gottesman, M. M. (1992) in *Molecular and Cellular Biology of Multidrug Resistance in Tumor Cells*, ed. Roninson, I. B. (Plenum, New York), pp. 91–106.
3. Ambudkar, S. V., Lelong, I. H., Zhang, J., Cardarelli, C. O., Gottesman, M. M. & Pastan, I. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 8472–8476.
4. Doige, C. A., Yu, X. & Sharom, F. J. (1992) *Biochim. Biophys. Acta* **1109**, 149–160.
5. Gill, D. R., Hyde, S. C., Higgins, C. F., Valverde, M. A., Mintenig, G. M. & Sepúlveda, F. V. (1992) *Cell* **71**, 23–32.
6. Sarkadi, B., Price, E. M., Boucher, R. C., Germann, U. A. & Scarborough, G. A. (1992) *J. Biol. Chem.* **267**, 4854–4858.
7. Valverde, M. A., Díaz, M., Sepúlveda, F. V., Gill, D. R., Hyde, S. C. & Higgins, C. F. (1992) *Nature (London)* **355**, 830–833.
8. Raviv, Y., Pollard, H. B., Bruggemann, E. P., Pastan, I. & Gottesman, M. M. (1990) *J. Biol. Chem.* **265**, 3975–3980.
9. Higgins, C. F. & Gottesman, M. M. (1992) *Trends Biochem. Sci.* **17**, 18–21.
10. Higgins, C. F. (1992) *Annu. Rev. Cell Biol.* **8**, 67–113.
11. Gottesman, M. M. & Pastan, I. (1993) *Annu. Rev. Biochem.* **62**, 385–427.
12. Ramu, A., Pollard, H. B. & Rosario, L. M. (1989) *Int. J. Cancer* **44**, 539–547.
13. Shalinsky, D. R., Jekunen, A. P., Christen, R. D., Kim, S., Khatibi, S. & Howell, S. B. (1993) *Br. J. Cancer* **67**, 30–36.
14. Altenberg, G. A., Young, G., Horton, J. K., Glass, D., Belli, J. A. & Reuss, L. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 9735–9738.
15. Chen, L. B. (1988) *Annu. Rev. Cell Biol.* **4**, 155–181.
16. Howell, N., Belli, T. A., Zaczkiewicz, L. T. & Belli, J. A. (1984) *Cancer Res.* **44**, 4023–4029.
17. Roninson, I. B., Abelson, H. T., Housman, D. E., Howell, N. & Varshavsky, A. (1984) *Nature (London)* **309**, 626–628.
18. Sognier, M. A., Zhang, Y., Eberle, R. L. & Belli, J. A. (1992) *Biochem. Pharmacol.* **44**, 1859–1868.
19. Thimmaiah, K. N., Horton, J. K., Qian, X.-d., Beck, W. T., Houghton, J. A. & Houghton, P. J. (1990) *Cancer Commun.* **2**, 249–259.
20. Butler, W. B. (1984) *Anal. Biochem.* **141**, 70–73.
21. McLaughlin, S. G. A. & Dilger, J. P. (1980) *Physiol. Rev.* **60**, 825–863.
22. Emaus, R. K., Grunwald, R. & Lemasters, J. J. (1986) *Biochim. Biophys. Acta* **850**, 436–448.
23. Duchen, M. R. & Biscoe, T. J. (1992) *J. Physiol. (London)* **450**, 33–61.
24. Piwnica-Worms, D., Chiu, M. L., Budding, M., Kronauge, J. F., Kramer, R. A. & Croop, J. M. (1993) *Cancer Res.* **53**, 977–984.
25. Choi, K., Frommel, T. O., Stern, R. K., Pérez, C. F., Kriegler, M., Tsuruo, T. & Roninson, I. B. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 7386–7390.
26. Nielsen, D. & Skovsgaard, T. (1992) *Biochim. Biophys. Acta* **1139**, 169–183.
27. Skovsgaard, T. (1978) *Cancer Res.* **38**, 1785–1791.
28. Homolya, L., Holló, Z., Germann, U. A., Pastan, I., Gottesman, M. M. & Sarkadi, B. (1993) *J. Biol. Chem.* **268**, 21493–21496.
29. Roepe, P. D. (1992) *Biochemistry* **31**, 12555–12564.