Biochemical and Biophysical Research Communications **276,** 231–237 (2000)
doi:10.1006/bbrc.2000.3459, available online at http://www.idealibrary.com on IDEAL®

Equivalent Death of P-Glycoprotein Expressing and Nonexpressing Cells Induced by the Protein Kinase C Inhibitor Staurosporine

Kellie M. Tainton, Astrid A. Ruefli, Mark J. Smyth, and Ricky W. Johnstone[1]
The Peter MacCallum Cancer Institute, Saint Andrews Place, East Melbourne 3002, Victoria, Australia

Received August 16, 2000

P-glycoprotein (P-gp) is an ATP-dependent drug pump that confers multidrug resistance. In addition to its ability to efflux toxins P-gp can also inhibit apoptosis induced by a wide array of cell death stimuli that rely on activation of intracellular caspases for full function. We have previously demonstrated that stimuli including drugs such as hexamethylene bisacetamide (HMBA), the cytotoxic lymphocyte granule protein granzyme B, and pore-forming proteins such as perforin, kill P-gp positive cells in a caspase-independent manner. We therefore hypothesised that drugs that are not effluxed by P-gp and which induce cell death in the absence of caspase activation could induce death of P-gp expressing cells. Staurosporine has been previously shown to kill cells in the absence of caspase activation. Consistent with our hypothesis, we demonstrate here that staurosporine can equivalently kill P-gp^{+ve} and P-gp^{-ve} tumor cell lines in a caspase-independent manner.

Key Words: **P-glycoprotein; staurosporine; apoptosis; caspase; chemotherapy; multidrug resistance.**

P-glycoprotein (P-gp), a member of the ATP-Binding Cassette (ABC) superfamily, is encoded by the *MDR1* gene in humans and *mdr1a* and *mdr1b* in mice and has been demonstrated to act as a very efficient toxin efflux molecule (1, 2). In the clinical setting, expression of P-gp on tumor cells confers resistance to a wide range of different chemotherapeutic agents constituting a multidrug resistant (MDR) phenotype and poor prognosis. The current working model maintains that P-gp removes xenotoxins in an energy (ATP)-dependent manner by intercepting the drug as it moves through the lipid membrane and flips the drug from the inner leaflet to the outer leaflet and into the extracellular media (3). Consistent with its toxin clearance role, P-gp is expressed on the surface of normal human cells found in the gut, liver, kidney tubules and at blood-tissue barriers (4). However, P-gp is also expressed in the adrenal gland, on hemopoietic stem cells, natural killer (NK) cells, antigen presenting dendritic cells and T and B lymphocytes (5, 6) and a role for P-gp in removing xenotoxins from these cells is not immediately apparent.

While the drug efflux function of P-gp has been well documented over many years, recent reports by our group and others have indicated that in addition to its role as an efflux pump, P-gp additionally regulates programmed cell death mediated by some chemotherapeutic drugs, serum starvation, ultraviolet (UV) irradiation and ligation of the cell surface death receptors Fas and tumor necrosis factor receptor (TNFR) (7, 8, 9). Common to these diverse apoptotic stimuli is their dependence on the activation of caspases to effectively mediate cell death. Indeed, we have previously shown that caspase-8 and -3 activation is inhibited by functional P-gp during Fas mediated apoptosis (8 and unpublished observations). Moreover, we have shown that HMBA, a hybrid polar compound that can induce terminal differentiation of transformed cells (10, 11), can induce death of P-gp expressing human tumor cells. In agreement with our earlier reports, HMBA induced activation of caspase-3 in P-gp^{-ve} but not P-gp^{+ve} cells. However, in the absence of caspase activation, HMBA could still induce cell death that was marked by the caspase-independent release of cytochrome c from the mitochondria to the cytosol and a reduction in Bcl-2 levels (12).

While functional P-gp can affect cell death induced by stimuli that rely on active caspases to efficiently kill cells, other stimuli such as HMBA, granzyme B and perforin, which are fully functional in the absence of caspase activation, are not affected by P-gp (8, 9, 12). We therefore hypothesised that the successful treatment of P-gp^{+ve} MDR tumors might be enhanced using chemotherapeutic agents that can function in the ab-

[1] To whom correspondence should be addressed. Fax: +61-3-9656-1411. E-mail: r.johnstone@pmci.unimelb.edu.au.

0006-291X/00 $35.00

sence of caspase activation. To illustrate this point, we set out to obtain agents that have been demonstrated to effectively kill target cells without the need for caspase activation. One such agent is staurosporine; a protein kinase C inhibitor that can kill cells even when intracellular caspases have been functionally inhibited (13, 14). In addition, staurosporine has been shown to inhibit drug efflux by P-gp (15, 16) and in some instances to reduce the expression of cell surface P-gp (17). We therefore reasoned that the combined actions of staurosporine would make it a likely candidate to effectively kill P-gp expressing cells.

We demonstrate here that staurosporine can efficiently induce equivalent caspase-independent death of both P-gp^{+ve} and parental P-gp^{-ve} T cell lines and LoVo colon carcinoma cells, without affecting the expression levels of P-gp. These data demonstrate that apoptotic agents that function in a caspase-independent manner do effectively kill P-gp expressing MDR cells.

MATERIALS AND METHODS

Cell culture. The acute T cell leukemia cell line, CEM-CCRF, its doxorubicin (DOX)-selected and resistant P-gp^{+ve} line CEM-A7^{+}, and the derived sublines 4G9 and 2G10 have been previously described (18). The T cell line 12D7 and production of 12D7-MDR1 cells by transduction with a retrovirus containing the MDR1 gene was described previously (19). A LoVo colon carcinoma cell line that constitutively expresses P-gp in the absence of drug treatment was also used. All cells were grown in RPMI medium 1640 supplemented with 10% (vol/vol) fetal calf serum, 2 mM glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin (GIBCO, Grand Island, NY). The 2G10 cells are grown in the above media supplemented with 5 ng/ml vincristine. The cell surface expression of P-gp was confirmed and monitored by fluorescence analysis using the anti-P-gp monoclonal antibody MRK 16 (Kamiya Biochemical Company, Thousand Oaks, CA). Drug efflux activities of P-gp and reversal with verapamil were assessed by rhodamine 123 exclusion assays as described (20). Cells were cultured for 16–72 h with 0–1000 nM staurosporine (Sigma, St. Louis, MO) or 50–1000 ng/ml vincristine (kindly donated by Dr Phillip Kantharidis, Peter MacCallum Cancer Institute, East Melbourne, Australia). To inhibit the activation of caspases, cells were pre-treated for 30–60 min with peptidyl fluoromethylketones (ZVAD-fmk, or Z-FAD-fmk as a control) (Enzyme System Products, Dublin, CA) (final 0–40 μM).

Viability assays. Cells were cultured at 2.5×10^5 cells/ml in the presence or absence of cell death stimuli for varying times. Trypan blue exclusion assays were performed as previously described (12). In all assays, cells from at least four different fields of view were counted for each data point and data were calculated as the mean ± SE and are representative of at least two separate assays. The number of apoptotic or dead cells (blue cells) was expressed as a percentage of the total cell number. TUNEL staining and ^{51}Cr release assays were performed as described (8, 21).

Clonogenic assays. Colony assays were performed on cells treated for 24 h with various apoptotic stimuli as previously described (9).

RESULTS AND DISCUSSION

Staurosporine can induce death of P-gp^{+ve} T cell lines. P-gp^{-ve} T cell leukemia cells (CCRF, 4G9, 12D7) and their P-gp^{+ve} partner cell lines (A7+, 2G10, 12D7-MDR1) have been used previously by our laboratory and others to study P-gp function (8, 9, 18, 19). In addition, we used a LoVo colon carcinoma cell line that constitutively expressed cell surface P-gp in the absence of drug treatment. Cells were assessed for P-gp expression, prior to and during the course of our assays, by fluorescence analysis using the anti-P-gp monoclonal antibody MRK 16 (Fig. 1). We tested the efflux function of P-gp in each cell line using rhodamine 123 (^{123}Rh) efflux assays (20, 22). P-gp expressing cell lines effectively effluxed ^{123}Rh and this function could be reversed by pretreatment of cells with 10 μM verapamil, a pharmacological inhibitor of P-gp function (data not shown). Addition of various doses (50–1000 nM) of staurosporine for 24 h resulted in equivalent death of P-gp^{+ve} and P-gp^{-ve} cells (Fig. 2). Cell death was initially measured by ^{51}Cr release from CEM/A7+ (Fig. 2A) and LoVo cells (Fig. 2D) or trypan blue viability assays on 4G9/2G10 cells (Fig. 2B), and 12D7/12D7-MDR1 cells (Fig. 2C), both of which measure death as a function of cell membrane integrity. In all cases, the P-gp^{+ve} cells were sensitive to staurosporine but were relatively insensitive to the chemotherapeutic drug vincristine which can be effluxed out of

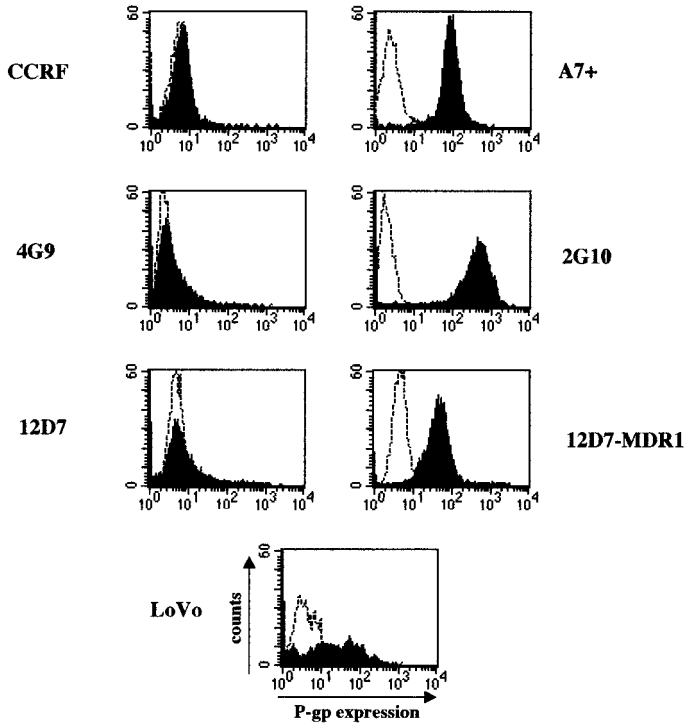

FIG. 1. Cell surface expression of P-gp. CCRF, 4G9, and 12D7 T cell lines and their P-gp expressing partner lines A7+, 2G10, and 12D7-MDR1 were used in this study. A LoVo colon carcinoma cell line that expressed P-gp in the absence of drug selection or genetic manipulation was also used. Cells were examined for expression of cell surface P-gp by immunofluorescence/flow cytometry using the MRK-16 monoclonal antibody (solid histogram) or an isotype control antibody (dashed line).

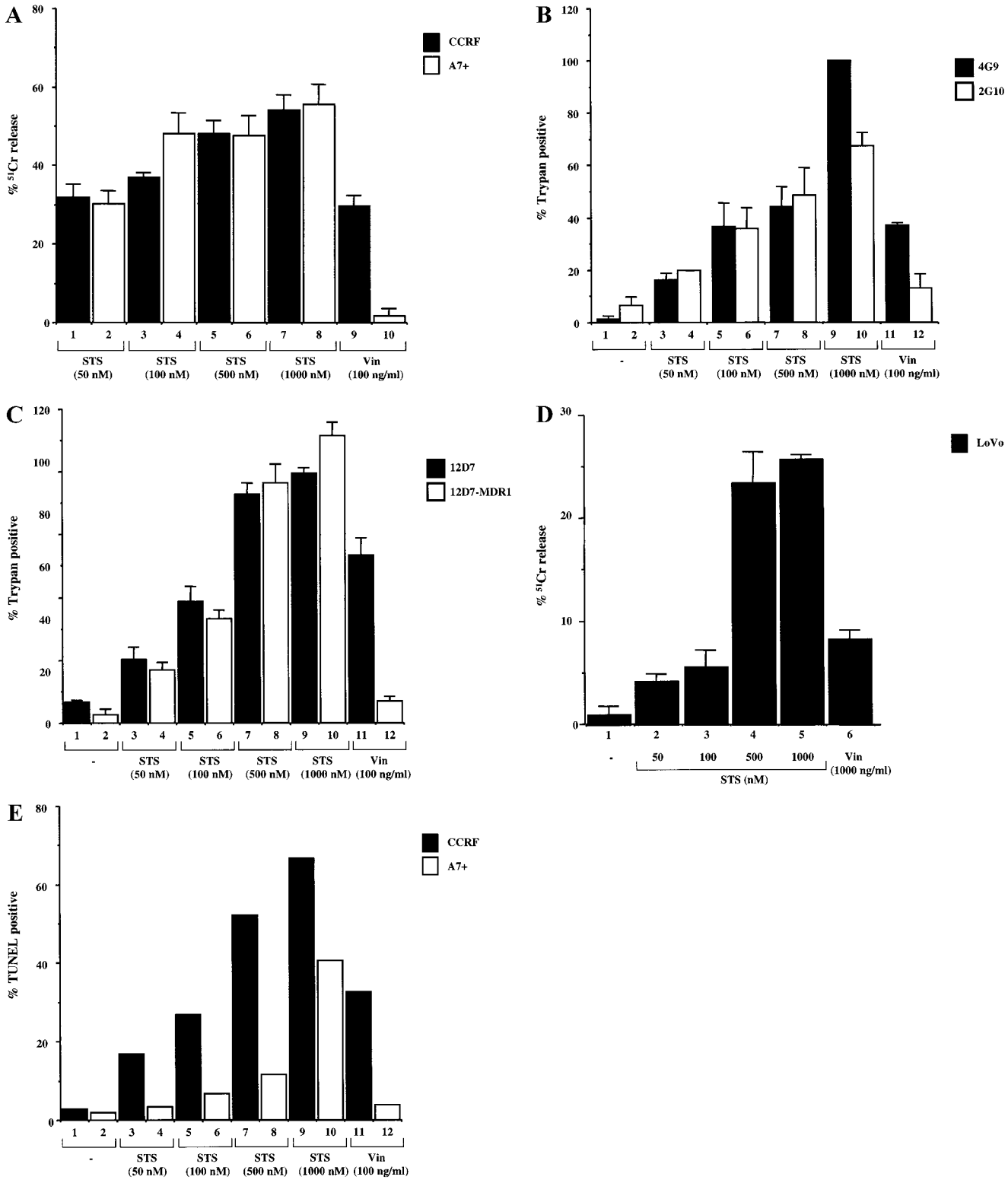

FIG. 2. Staurosporine induces equivalent death of P-gp^{-ve} and P-gp^{+ve} tumor cell lines. P-gp^{+ve} and P-gp^{-ve} T lymphocyte and colon carcinoma cell lines were cultured for 24 h with 0–1000 nM staurosporine (STS) or 100 ng/ml vincristine (Vin). Cell death was assessed by ^{51}Cr release (A, D) or trypan blue exclusion (B, C) assays as described under Materials and Methods. Data are calculated as the mean ± SE and are representative of at least two different experiments. (E) DNA fragmentation of CCRF and A7+ cells treated with staurosporine or vincristine for 24 h was assessed by TUNEL staining. Shown is representative of seven separate experiments demonstrating a similar effect.

cells by P-gp (23) and is reliant on active caspases for full apoptotic function (9). These data demonstrate that while P-gp is fully functional in the P-gp^{+ve} A7+, 2G10, 12D7-MDR1, and LoVo cell lines, expression of P-gp did not inhibit death induced by staurosporine.

We also tested CCRF and A7+ cells for DNA damage following treatment with staurosporine or vincristine by TUNEL assay. As shown in Fig. 2E, in contrast to the ^{51}Cr release or trypan viability tests, staurosporine reproducibly produced less DNA damage in P-gp^{+ve} cells compared to parental P-gp^{-ve} cells. Similar results were seen in the other cell lines tested (data not shown). This is consistent with the findings of Deas *et al.* (13) who demonstrated that staurosporine produced caspase-dependent nuclear damage and caspase-independent cytosolic changes in T lymphocytes, and our previous studies showing that P-gp expression correlates with a decrease in caspase activity in these cells (8, 9, 12). We have also demonstrated that although nuclear events such as DNA damage and chromatin condensation might be compromised in P-gp^{+ve} cells treated with certain stimuli such as granzyme B or HMBA, damage to the cell membrane which can occur in the absence of caspase activation, correlated with death of cells as assessed by sensitive long term clonogenic assays (9, 12).

To demonstrate that staurosporine could equivalently kill P-gp^{+ve} and P-gp^{-ve} cells with similar kinetics, 4G9 and 2G10 cells were treated with 50 nM, 125 nM or 500 nM staurosporine, or 50 ng/ml vincristine and death was assessed over a three day time course (Figs. 3A and 3B). Both 4G9 and 2G10 cells were sensitive at each time point to staurosporine-induced apoptosis over a range of concentrations, however only the P-gp^{-ve} 4G9 cells were sensitive to death induced by vincristine. These data confirm that staurosporine induces equivalent cell membrane perturbations in P-gp^{+ve} and P-gp^{-ve} T cell lines at various doses over a three day time course. Staurosporine and its analogues have been demonstrated to inhibit P-gp function (15, 16) and it appears to do so by inhibiting drug binding to P-gp rather than by inhibiting PKC-mediated phosphorylation of P-gp (24, 25, 26). We therefore determined whether inhibition of P-gp function by the pharmacological inhibitor verapamil affected staurosporine-mediated death of P-gp^{+ve} cells. As shown in Fig. 3, pretreatment of cell lines with verapamil did not alter the sensitivity of these cells to staurosporine-induced cell death. By contrast, P-gp^{+ve} cells were more sensitive to vincristine-mediated cell death following pretreatment with verapamil indicating that P-gp function could be inhibited at this dose of verapamil. This demonstrates that functional P-gp in no way affects the cytotoxic activity of staurosporine.

Although ^{51}Cr release and trypan blue have often been used as cell death parameters, we performed sensitive clonogenic assays on T cell lines treated with

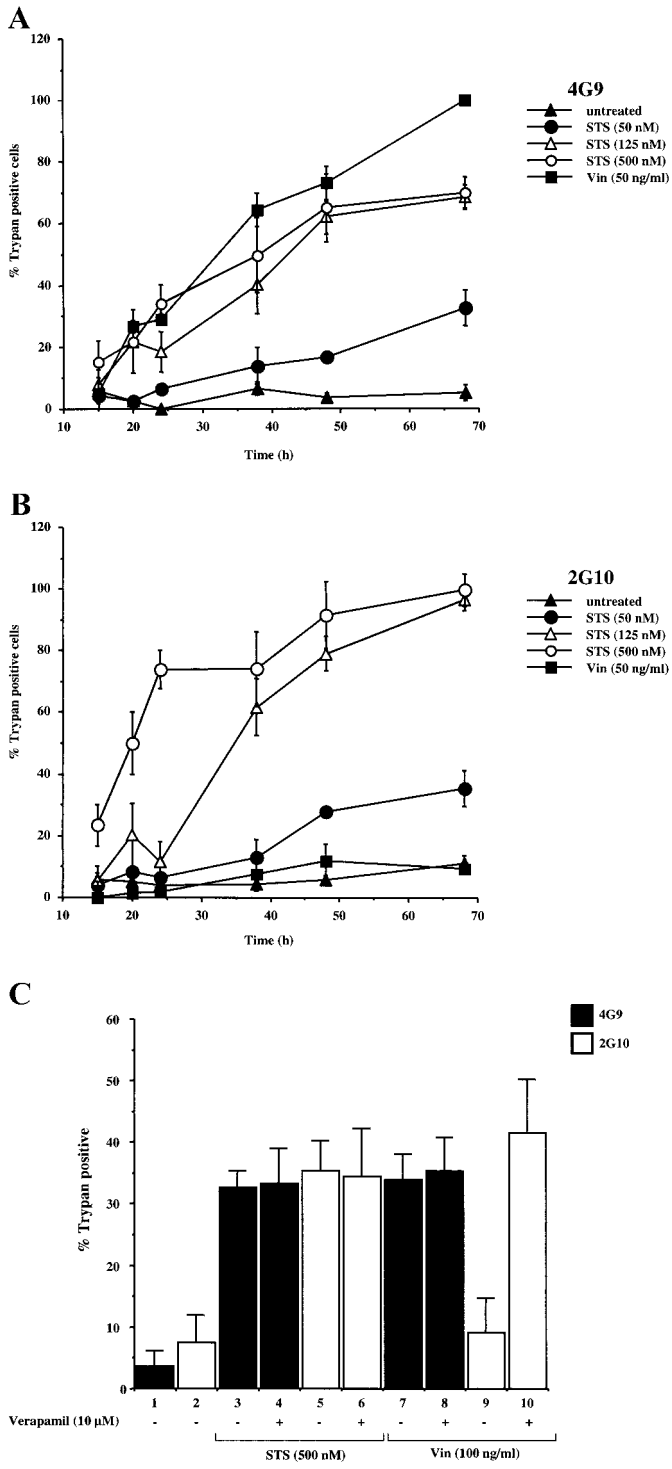

FIG. 3. Staurosporine kills P-gp^{-ve} and P-gp^{+ve} cells with similar kinetics and is not influenced by verapamil. 4G9 (A) and 2G10 (B) cells were treated with 50–500 nM staurosporine (STS) or 50 ng/mg vincristine (Vin) for 15–68 h. (C) The effect of inhibition of P-gp function on staurosporine-induced cell death was assessed by addition of 10 μM verapamil. 4G9 and 2G10 cells were treated with staurosporine or vincristine in the presence or absence of verapamil. In all assays cell death was measured by trypan blue exclusion assays. Data are calculated as the mean ± SE and are representative of at least two different experiments.

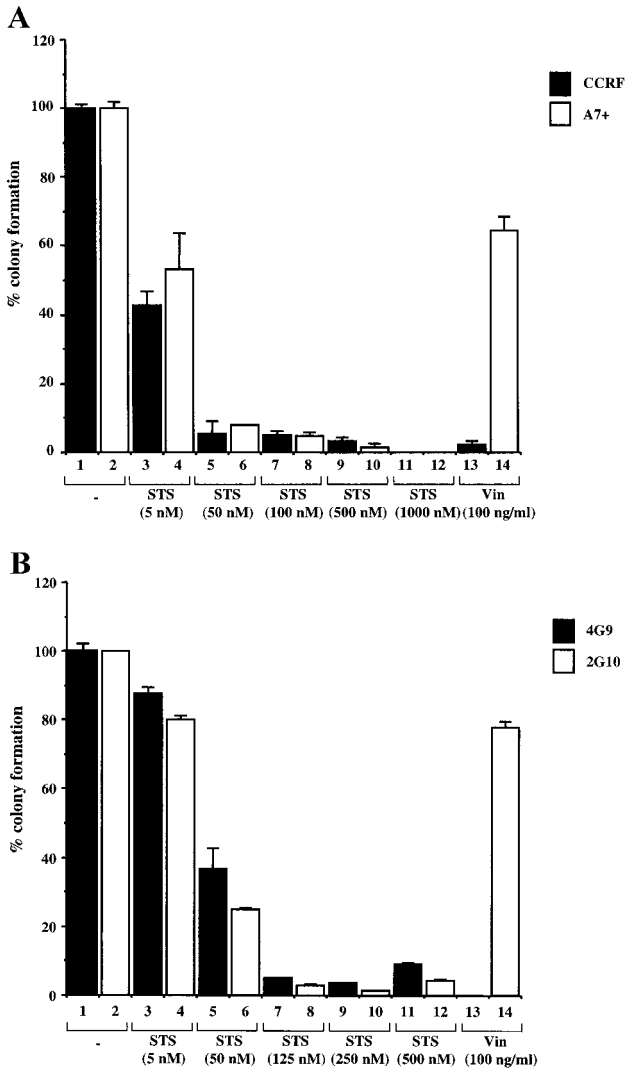

FIG. 4. Staurosporine induces equivalent death of P-gp^{-ve} and P-gp^{+ve} cells as assessed by sensitive clonogenic assays. After 24 h in culture with 5–1000 nM staurosporine (STS) or 100 ng/ml vincristine (Vin), A7+ and CCRF (A), or 2G10 and 4G9 (B) cells were plated out in soft agar. Colonies were scored after 14 days in culture as described under Materials and Methods.

staurosporine to confirm our findings. Briefly, P-gp^{+ve} and P-gp^{-ve} cells were treated with staurosporine (5–1000 nM) or vincristine (100 ng/ml) for 24 h then plated out in soft agar, grown at 37°C for 14 days at which point, colonies were counted. As shown in Figs. 4A and 4B, treatment of P-gp^{-ve} and P-gp^{+ve} cell lines with staurosporine resulted in a dose-dependent loss of colony formation while vincristine only inhibited formation of colonies containing P-gp^{-ve} CCRF and 4G9 cells. These data confirm the ^{51}Cr release and trypan blue exclusion assays demonstrating equivalent staurosporine induced death of P-gp^{+ve} and P-gp^{-ve} cells.

Staurosporine does not alter P-gp expression. It has been previously demonstrated that addition of staurosporine to P-gp^{+ve} cells results in a significant decrease in P-gp mRNA and protein levels (17). We tested the effect of staurosporine on P-gp expressed on 2G10 cells by fluorescence analysis (Fig. 5A). Cells treated with staurosporine (500 nM) for 24 h were assessed for P-gp expression using the specific MRK 16 anti-P-gp monoclonal antibody with both live and dying cells (as assessed by forward and side scatter profiles) included in the analysis. As can be seen, treatment with stauro-

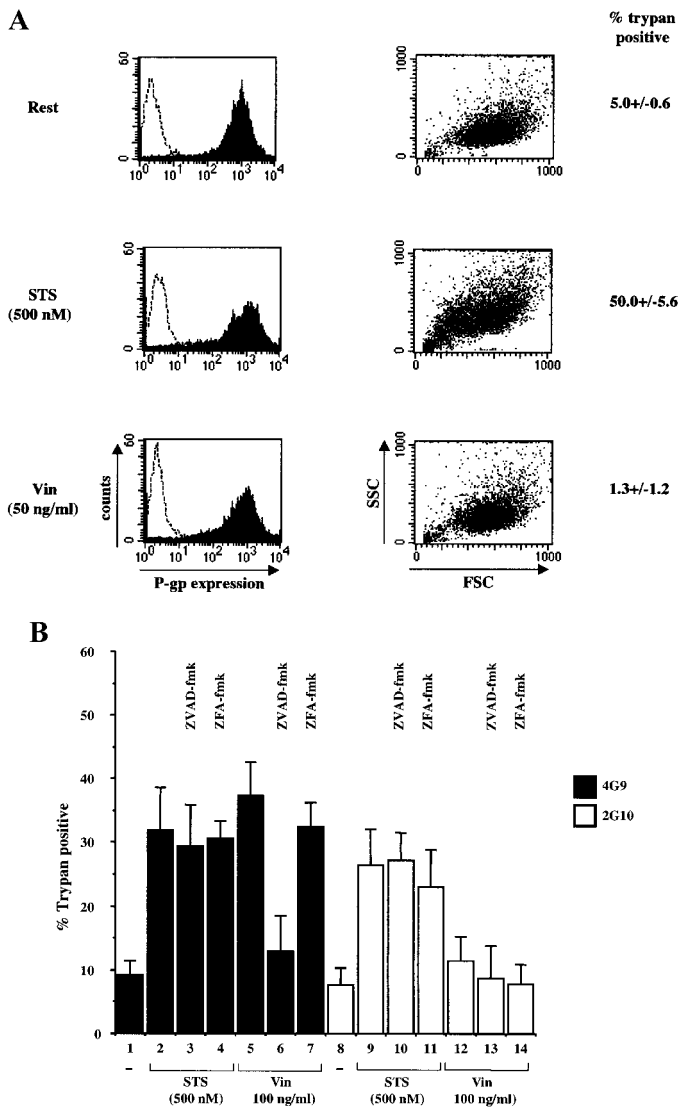

FIG. 5. Staurosporine does not affect expression of P-gp and induces caspase-independent cell death. (A) 2G10 cells were treated with 500 nM staurosporine (STS) or 50 ng/ml vincristine (Vin) for 24 h. P-gp expression was assessed by immunofluorescence/flow cytometry using the MRK-16 monoclonal antibody (dashed line) or an isotype control antibody (solid histogram). Cell death was determined by trypan blue exclusion assays and can be visualised by altered forward and side scatter flow cytometry profiles. (B) 4G9 and 2G10 cells were treated with 500 nM staurosporine (STS) or 100 ng/ml vincristine (Vin) for 24 h. In some wells, cells were preincubated for 30–60 min with 40 μM ZFA-fmk or ZVAD-fmk. Cells were assayed by trypan blue exclusion assays.

sporine did not significantly alter cell surface expression of P-gp on 2G10 cells although these cells were clearly undergoing cell death as assessed by the accompanying forward and side scatter profiles and trypan blue exclusion assays.

Staurosporine induces caspase-independent death of P-gp^{+ve} T cell lines. We have previously demonstrated that cell death stimuli such as granzyme B (8), perforin (9) and HMBA (12) can equivalently kill P-gp^{+ve} and P-gp^{-ve} cells in the absence of caspase activation. It has been shown that staurosporine can induce caspase-independent death of primary human T cells (13) and we therefore sought to determine whether staurosporine induced caspase-independent death of our P-gp^{+ve} and P-gp^{-ve} cell lines. Cells were pretreated with the caspase inhibitory peptide ZVAD-fmk or control chymotrypsin inhibitory peptide ZFA-fmk for 30–60 min before addition of staurosporine (250 nM) or vincristine (100 μg/ml) for 24 h and cell viability was assessed by trypan blue exclusion assays (Fig. 5B). Addition of ZVAD-fmk or ZFA-fmk had little or no effect on cell death induced by staurosporine in either P-gp^{+ve} or P-gp^{-ve} cells (Fig. 5B, lanes 3, 4, 10, 11). However, as we have previously demonstrated, death of P-gp^{-ve} cells induced by vincristine was significantly inhibited by the caspase-specific peptide ZVAD-fmk but not by the control ZFA-fmk peptide (Fig. 3B, lanes 6, 7). These data demonstrate that staurosporine induces equivalent caspase-independent death of P-gp^{+ve} and P-gp^{-ve} cell lines.

We have previously shown that expression of functional P-gp correlated with a loss of caspase activation upon apoptosis induction and that stimuli that can effectively kill P-gp expressing cells can do so in the absence of caspase activation (8, 9, 12). Based on these findings, we predicted that cytotoxic agents that function in the absence of active caspases and that are not effluxed by P-gp would be able to kill P-gp^{+ve} cells. The data presented here supports that hypothesis with staurosporine capable of equivalently killing P-gp^{+ve} and P-gp^{-ve} cells via a caspase-independent mechanism. Staurosporine has been demonstrated to disrupt the mitochondrial membrane potential in the absence of active caspases resulting in release of cytochrome c into the cytosol and ultimately cell death (27). We have observed similar effects with HMBA (12) and since both staurosporine (27)- and HMBA (12.28)-induced cell death can be blocked by overexpression of Bcl-2 which functions to maintain mitochondrial membrane potential, it is possible that other caspase-independent cell death stimuli will function by inhibiting mitochondrial function.

Our findings may have relevance to the potential treatment of P-gp expressing MDR tumors. Based on our data, chemotherapeutic agents that function in a caspase-independent manner would be more effective than those that rely on caspases for full activity. Dissecting the molecular pathways leading to caspase-independent cell death and understanding how these pathways may be triggered and inhibited could lead to novel chemotherapeutic agents for the treatment of MDR tumors.

ACKNOWLEDGMENTS

R.W.J. is a Wellcome Trust Senior Research Fellow and M.J.S. is a Principal Research Fellow, Australian National Health and Medical Research Council (NH&MRC). The work was supported by Project Grants from the NH&MRC and the Anti-Cancer Council, Victoria.

REFERENCES

1. Ueda, K., Cardarelli, C., Gottesman, M. M., and Pastan, I. (1987) Expression of a full length cDNA for the human MDR1 gene confers resistance to colchicine, doxorubicin, and vinblastine. *Proc. Natl. Acad. Sci. USA* **84,** 3004–3008.
2. Gottesman, M. M., and Pastan, I. (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter. *Ann. Rev. Biochem.* **62,** 385–427.
3. Higgins, C. F., and Gottesman, M. M. (1997) Is the multidrug transporter a flippase? *Trends Biochem. Sci.* **17,**18–21.
4. Cordon-Cardo, C., O'Brien, J. P., Boccia, J., Casals, D., Bertino, J. R., and Melamed, M. R. (1990) Expression of the multidrug resistance gene product (P-glycoprotein) in human normal and tumor tissues. *J. Histochem. Cytochem.* **38,**1277–1287.
5. Klimecki, W. T., Futscher, B. W., Grogan, T. M., and Dalton, W. S. (1994) P-glycoprotein expression and function in circulating blood cells from normal volunteers. *Blood* **83,** 2451–2458.
6. Randolph, G. J., Beaulieu, S., Pope, M., Sugawara, I., Hoffman, L., Steinman, R. M., and Muller, W. A. (1998) A physiologic function for p-glycoprotein (MDR-1) during the migration of dendritic cells from skin via afferent lymphatic vessels. *Proc. Natl. Acad. Sci. USA* **95,** 6924–6929.
7. Robinson, L. J., Roberts, W. K., Ling, T. T., Lamming, D., Sternberg, S. S., and Roepe, P. D. (1997) Human MDR1 protein overexpression delays the apoptotic cascade in Chinese hamster ovary fibroblasts. *Biochemistry* **36,**11169–11178.
8. Smyth, M. J., Krasovskis, E., Sutton, V. R., and Johnstone, R. W. (1998) The drug efflux protein, P-glycoprotein, additionally protects drug-resistant tumor cells from multiple forms of caspase-dependent apoptosis. *Proc. Natl. Acad. Sci. USA* **95,** 7024–7029.
9. Johnstone, R. W., Krasovskis, E., and Smyth, M. J. (1999) Inhibition of caspase-dependent apoptosis by P-glycoprotein. *Blood* **93,** 1075–1085.
10. Marks, P. A., Richon, V. M., Kiyokawa, H., and Rifkind, R. A. (1994) Inducing differentiation of transformed cells with hybrid polar compounds: a cell cycle-dependent process. *Proc. Natl. Acad. Sci. USA* **91,** 10251–10256.
11. Richon, V. M., Russo, P., Venta-Perez, G., Cordon-Cardo, C., Rifkind, R. A., and Marks, P. A. (1997) Two cytodifferentiation agent-induced pathways, differentiation and apoptosis, are distinguished by the expression of human papillomavirus 16 E7 in human bladder carcinoma cells. *Cancer Res.* **57,** 2789–2797.
12. Ruefli, A. A., Smyth, M. J., and Johnstone, R. W. (2000) HMBA induces activation of a caspase-independent cell death pathway to overcome P-glycoprotein-mediated multidrug resistance. *Blood* **95,** 2378–2385.
13. Deas, O., Dumont, C., MacFarlane, M., Rouleau, M., Hebib, C., Harper, F., Hirsch, F., Charpentier, B., Cohen, G. M., and Senik,

A. (1998) Caspase-independent cell death induced by anti-CD2 or staurosporine in activated human peripheral T lymphocytes. *J. Immunol.* **161,** 3375–3383.

14. Amarante-Mendes, G. P., Finucane, D. M., Martin, S. J., Cotter, T. G., Salvesen, G. S., and Green, D. R. (1998) Anti-apoptotic oncogenes prevent caspase-independent commitment for cell death. *Cell Death Differ.* **5,** 298–306.
15. Sato, W., Yusa, K., Naito, M., and Tsuruo, T. (1990) Staurosporine, a potent inhibitor of C-kinase, enhances drug accumulation in multidrug resistant cells. *Biochem. Biophy. Res. Commun.* **173,** 1252–1257.
16. Ma, L. D., Marquardt, D., Takemoto, L., and Center, M. S. (1991) Analysis of P-glycoprotein phosphorylation in HL60 cells isolated for resistance to vincristine. *J. Biol. Chem.* **266,** 5593–5599.
17. Sampson, K. E., Wolf, C. L., and Abraham, I. (1993) Staurosporine reduces P-glycoprotein expression and modulates multidrug resistance. *Cancer Lett.* **68,** 7–14.
18. Zalcberg, J. R., Hu, X. F., Wall, D. M., Mirski, S., Cole, S., Nadalin, G., De Luise, M., Parkin, J. D., Vrazas, V., Campbell, L., and Kantharidis, P. (1994) Cellular and karyotypic characterization of two doxorubicin resistant cell lines isolated from the same parental human leukemia cell line. *Int J Cancer* **57,** 522–528.
19. Lee, C. G. L., Pastan, I., and Gottesman, M. M. (1996) Retroviral transfer of human MDR1 gene into human T lymphocytes. *Meth. Enzymol.* **292,** 557–572.
20. Yamamoto, T., Iwasaki, T., Watanabe, N., Oshimi, K., Naito, M., Tsuruo, T., and Kobayashi, Y. (1993) Expression of multidrug resistance P-glycoprotein on peripheral blood mononuclear cells of patients with granular lymphocyte-proliferative disorders. *Blood* **81,** 1342–1346.
21. Trapani, J. A., Jans, P., Smyth, M. J., Froelich, C. J., Williams, E. A., Sutton, V. R., and Jans, D. A. (1998) Perforin-dependent nuclear entry of granzyme B precedes apoptosis, and is not a consequence of nuclear membrane dysfunction. *Cell Death Diff.* **5,** 488–493.
22. Efferth, T., Lohrke, H., and Volm, M. (1989) Reciprocal correlation between expression of P-glycoprotein and accumulation of rhodamine 123 in human tumors. *Anticancer Res.* **9,** 1633–1637.
23. Cass, C. E., Janowska-Wieczorek, A., Lynch, M. A., Sheinin, H., Hindenburg, A. A., and Beck, W. T. (1989) Effect of duration of exposure to verapamil on vincristine activity against multidrug-resistant human leukemic cell lines. *Cancer Res.* **49,** 5798–5804.
24. Miyamoto, K., Inoko, K., Wakusawa, S., Kajita, S., Hasegawa, T., Takagi, K., and Koyama, M. (1993) Inhibition of multidrug resistance by a new staurosporine derivative, NA-382, in vitro and in vivo. *Cancer Res.* **53,** 1555–1559.
25. Smith, C. D., and Zilfou, J. T. (1995) Circumvention of P-glycoprotein-mediated multiple drug resistance by phosphorylation modulators is independent of protein kinases. *J. Biol. Chem.* **270,** 28145–18152.
26. Budworth, J., Davies, R., Malkhandi, J., Gant, T. W., Ferry, D. R., and Gescher, A. (1996) Comparison of staurosporine and four analogues: their effects on growth, rhodamine 123 retention and binding to P-glycoprotein in multidrug-resistant MCF-7/Adr cells. *Br. J. Cancer* **73,** 1063–1068.
27. Bossy-Wetzel, E., Newmeyer, D. D., and Green, D. R. (1998) Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization. *EMBO J.* **17,** 37–49.
28. Siegel, D. S., Zhang, X., Feinman, R., Teitz, T., Zelenetz, A., Richon, V. M., Rifkind, R. A., Marks, P. A., and Michaeli, J. (1998) Hexamethylene bisacetamide induces programmed cell death (apoptosis) and down-regulates BCL-2 expression in human myeloma cells. *Proc. Natl. Acad. Sci. USA* **95,** 162–167.