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THE LEUKOTRIENE LTD_4 RECEPTOR ANTAGONIST MK571 SPECIFICALLY MODULATES MRP ASSOCIATED MULTIDRUG RESISTANCE

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Summary. The multidrug resistant cell lines HL60/AR and GLC4/ADR show high overexpression of the gene encoding the multidrug resistance associated protein MRP compared to their drug sensitive parental counterparts. This and the virtual absence of mdr1/P-glycoprotein gene expression was proven by a complementary DNA polymerase chain reaction (cDNA-PCR) approach. Applying a 72-hour tetrazolium based colorimetric MTT-assay we demonstrate on both MDR sublines a dose-dependent modulation of drug resistances by the leukotriene LTD_4 receptor antagonist MK571. A complete reversal of vincristine resistances was achieved at final MK571 concentrations of 30 μM (HL60/AR) or 50 μM (GLC4/ADR) which by itself did not disturb cellular proliferation. The drug resistance of a mdr1/P-gp overexpressing multidrug-resistant HL60 subline, in contrast, was not significantly affected by MK571. Similar effects were seen using the glutathione (GSH) synthesis inhibitor buthionine sulfoximine (BSO). Our results point to a relationship between MRP and a conjugate transporter and identify MK571 as a new tool structure for developing modulators specific for a MRP associated multidrug resistance.

Cellular multidrug resistances (MDR) mediated by the 170 kDa (M_r) mdr1/P-glycoprotein (mdr1/P-gp) were intensely studied in recent years (1), and the clinical occurrence of this type of MDR is suggested by numerous reports (2). In the meantime, a series of compounds (chemosensitizers, modulators) has been identified which block P-gp linked efflux of drugs very efficiently. Some of these drugs are investigated for their MDR reversal potency in clinical phase II trials (3). Another member of the ABC transporter super gene family (ABC = ATP binding cassette), the 190 kDa (M_r) multidrug resistance associated protein (MRP) was identified and characterized recently (4-6). Little information is available on the clinical significance of a MRP associated MDR. Nonetheless, we have demonstrated distinct increases of MRP together with mdr1/P-gp gene expression in chemotherapeutically treated cancers (7-9). Compounds modulating a MRP associated MDR are rare at present. Some agents, however, which originally

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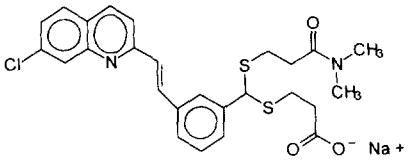

Figure 1. Structure of MK571.

were found to modulate a P-gp mediated MDR efficiently show some activity, such as the Ca^{2+} antagonistic phenylalkylamine verapamil (10-13), or the immunosuppressive cyclic peptide cyclosporin A and its non-immunosuppressive derivative SDZ PSC 833 (13). By using the γ-glutamylcysteine synthetase inhibitor BSO (14) another approach to MDR modulation was pursued. Thereby, the anthracycline resistance of MDR cells could be influenced most likely via depletion of GSH (15, 16). In particular, such effects of BSO were shown on the non-P-gp MDR cell lines HL60/AR (17) or GLC4/ADR (18) which now have been identified as MRP overexpressing ones. This points to a relationship between GSH linked funtions and the MRP drug transporter. While it is still unclear at present whether anticancer drugs like vincristine or adriamycin are transformed to GSH conjugates in tumor cells (19), leukotrienes definitely are at least partly effluxed as GSH conjugates from cells (20). In fact, it was recently demonstrated that leukotriene LTC_4 - the GSH conjugate of leukotriene LTA_4 - is transported in an ATP-dependent manner into membrane vesicles prepared from mouse mastocytoma cells (21), from MRP overexpressing multidrug resistant HL60/ADR cells (22), or from HeLa MRP transfectants (23). The LTC_4 transport into these vesicles was competitively inhibited by the leukotriene LTD_4 receptor antagonist MK571, an anionic quinoline derivative (structure shown in Fig. 1) (21-23). Moreover, photoaffinity labeling using [^{3}H]LTC_4 identified the distinct overexpression of a 190 kDa protein species in membranes prepared from these cell lines, and the labeling was efficiently competed by MK571 (21-23). Altogether, these results suggest that MRP represents a transporter of GSH conjugates, and prompted us to study the biological activity of MK571 for reversal of MRP or P-gp associated cellular drug resistances.

Materials and Methods

Cell Lines. The MDR sublines of the human promyelocytic leukemia cell line (ATCC CCL 240), i.e. the daunomycin selected, P-gp overexpressing subline HL60/DAU120, and the non-P-gp MDR cell line HL60/AR (24) were cultured in the presence of 150 ng/ml or 56 ng/ml daunomycin, respectively. The MDR subline GLC4/ADR (10) derived from the human small cell lung cancer cell line GLC4 was maintained in the presence of 680 ng/ml adriamycin. All cell lines were cultured under regular conditions in RPMI1640 medium and the usual supplements including 10% fetal calf serum.
Drugs. Vincristine-sulfate (VCR), doxorubicin-HCl (adriamycin, ADR), daunomycin-HCl (DAU), and L-buthionine-[*S,R*]-sulfoximine (BSO) were purchased from Sigma (Deisenhofen/Germany). The LTD_4 receptor antagonist MK571 (identical with L-660-711: sodium salt of (±)-3-[[[3- [2- (7-chloro-2-quinolinyl)- (*E*) -ethenyl]phenyl] [[3-

(dimethylamino)- 3-oxopropyl]thio]methyl]thio] propionic acid) was synthesized at the chemical department of Byk Gulden according to (25).

PCR Gene Expression Analysis. The preparation of total cellular RNA, the synthesis of cDNA using random hexanucleotide primers (Boehringer Mannheim, Germany) and RAV2 reverse transcriptase (Amersham, Braunschweig, Germany), and the cDNA-PCR using mdr1 specific amplimers (expected size of the amplified material: 229 bp) were performed as described (26). The conditions for PCR were modified according to (9). The following amplimers were used for expression analysis of the MRP gene (expected size 326 bp; 5'-CGT GTA CTC CAA CGC TGA C-3' (sense) and 5'-CTG GAC CGC TGA CGC CCG TGA C-3' (antisense)), or the GAPDH gene (glyceraldehyde-3-phosphate-dehydrogenase; expected size 358 bp; 5'-CGG GAA GCT TGT GAT CAA TGG-3' (sense) and 5'-GGC AGT GAT GGC ATG GAC TG-3' (antisense)). The latter was included as a control for the amount of cDNA present in the samples. The amplimers were established using the published cDNA sequences (5, 27). The amplimers used for MRP gene expression analysis were recently proven to be specific for the MRP gene by restriction digest analysis (8). The signal intensities were evaluated by the CS-1 Videoimager (Cybertech, Berlin/Germany), and normalized to the signal intensities obtained using the GAPDH specific amplimers.

Drug Sensitivity Testing. Prior to analysis the MDR sublines were cultivated without the drug used for selection for about one to two weeks. The tetrazolium based colorimetric MTT-assay (28) was applied for determination of drug sensitivities of the cell lines in the absence or presence of modulators. Therefore, cell aliquots, i.e. 40000 cells/well in case of HL60 and sublines, or 24000 cells/well in case of GLC4 and the subline GLC4/ADR, were seeded in triplicate into 96-well microtiter plates and incubated for 72 hours without or with the chosen mixtures of drugs. The tetrazolium salt was then added to a final concentration of 0.5 mg/ml. After incubation for 4 hours, DMSO was added, and the plates were vigorously shaken for 1 hour to dissolve the formazan crystals. Absorption was measured at 540 nm using an automatic microplate reader.

Results

Fig. 2 shows a cDNA-PCR analysis for mdr1/P-gp (28 cycles), MRP (28 cycles), or GAPDH (21 cycles) gene expression in the MDR sublines HL60/DAU120, HL60/AR, GLC4/ADR, and the parental cell lines HL60 and GLC4. Except in case of HL60/DAU120 cells where a strong mdr1 amplification signal was observed, virtually no mdr1 gene expression could be identified in the other cell lines used. In contrast, a basal expression of the MRP gene was found throughout. However, in the sublines HL60/AR and GLC4/ADR a distinct overexpression of the MRP gene occurs. For a better evaluation of the extent of MRP gene expression in the samples, we performed the cDNA-PCR by varying the numbers of PCR cycles (Fig. 3) which revealed an almost identical overexpression in the cell lines HL60/AR and GLC4/ADR, and very similar levels in the cell lines HL60, HL60/DAU120, and GLC4.

The non-P-gp MDR cell lines used in the present work showed distinct relative resistances to anticancer drugs (10, 24). This and the relative resistances of the P-gp overexpressing subline HL60/DAU120 were tested by MTT-assays. Calculating the ratios of IC_{50} values of the MDR sublines and the parental cell lines, we measured relative resistances towards vincristine of 38-fold (HL60/AR), 23-fold (GLC4/ADR), and 272-fold (HL60/DAU120), towards adriamycin of 99-fold (HL60/AR), 683-fold (GLC4/ADR), and 100-fold (HL60/DAU120), respectively. There are clearly different

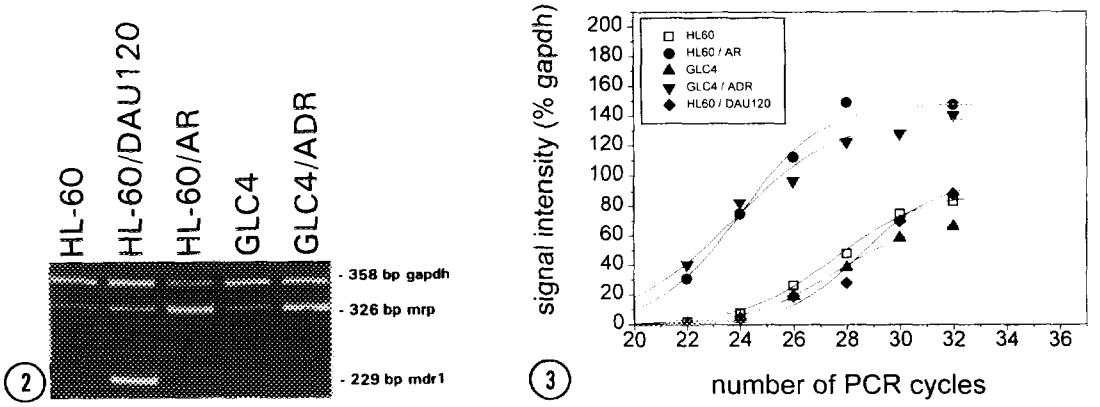

Figure 2. Expression analysis of the mdr1/P-gp, MRP and GAPDH genes by cDNA-PCR.

Figure 3. Kinetics of MRP gene expression analysis by cDNA-PCR.

ratios of vincristine to adriamycin resistance values between the MRP and P-gp overexpressing MDR cell lines. The cytotoxic/antiproliferative activity of the compounds MK571 or BSO, respectively, either alone or in combination with vincristine or adriamycin were also examined by MTT-assays. MK571 was rather untoxic to the cells showing IC_{50} values between 70 μM and 90 μM on all cell lines tested. Using BSO no IC_{50} was reached at final drug concentrations of up to 100 μM except on the MRP overexpressing subline HL60/AR where an IC_{50} value of about 8 μM was monitored (data not shown; the effects of the chemosensitizers alone at fixed concentrations, however, are depicted in the Figs. 4 and 5 at the y-axes). Fig. 4A shows the dose dependent reversal of the vincristine resistance of the subline GLC4/ADR by MK571. A significant effect of the chemosensitizer is already seen at a concentration of 10 μM.

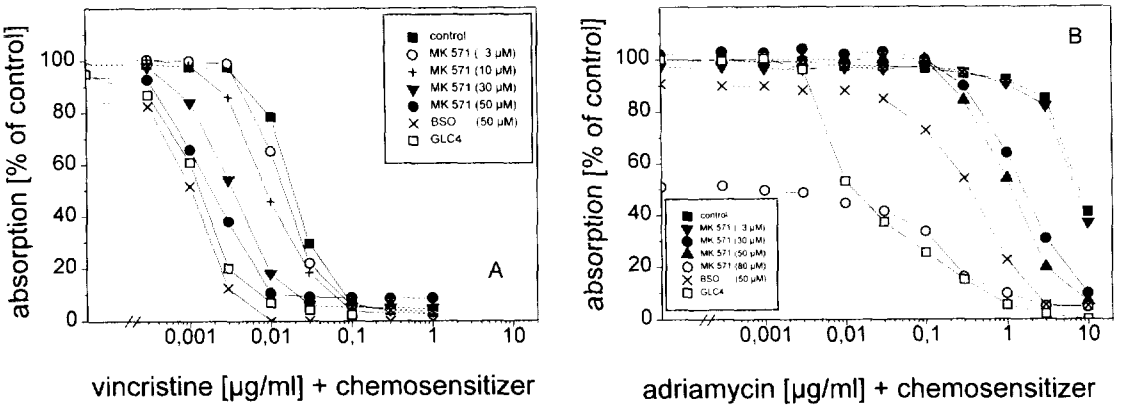

Figure 4. Effects of MK571 or BSO on (A) vincristine or (B) adriamycin sensitivities of the MRP overexpressing MDR subline GLC4/ADR (72 hours MTT-assays).

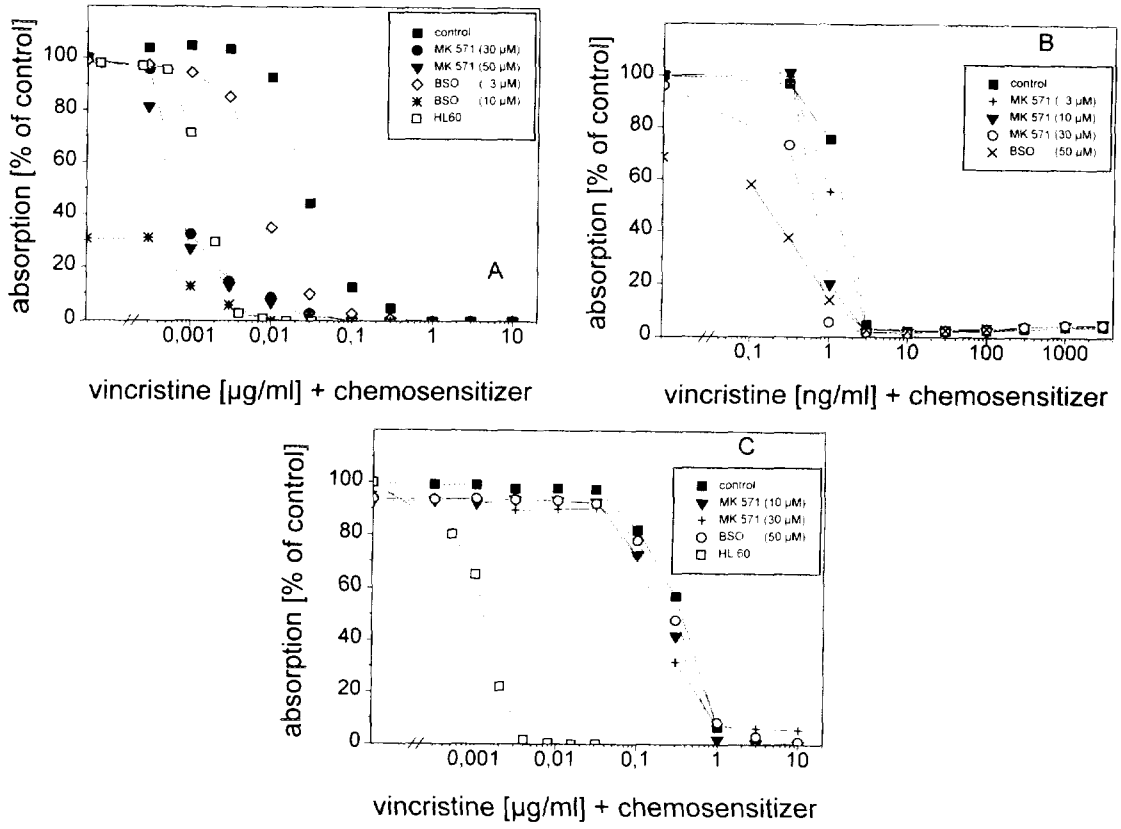

Figure 5. Effects of MK571 or BSO on vincristine sensitivities of (A) the MRP overexpressing MDR subline HL60/AR, (B) the parental cell line HL60, or (C) the mdr1/P-gp overexpressing MDR subline HL60/DAU120.

Using 50 μM of MK571 which is still untoxic to the cells, the sensitivity of the parental cell line is reached. The same result was found using 50 μM BSO. In case of adriamycin, the resistance modulation appears to be incomplete (Fig. 4B). Nonetheless, after addition of higher concentrations of MK571 a drastic shift of the adriamycin response curve is seen. Similar results were observed using the MRP overexpressing subline HL60/AR where the vincristine sensitivity is completely restored by 30 μM MK571 (Fig. 5A). Interestingly, BSO produced here a significant resistance modulation already at a final concentration of 3 μM. The vincristine sensitivity of the parental HL60 cell line was only slightly enhanced by 30 μM MK571. Applying 50 μM BSO, however, this effect appeared to be somewhat stronger (Fig. 5B). A similar result was seen using the small cell lung cancer cell line GLC4 (data not shown). This might be explained by the residual expression of the MRP gene in these parental cell lines (see Figs. 2 and 3). Remarkably, under the same conditions the vincristine resistance of the P-gp overexpressing subline HL60/DAU120 is only marginally influenced by MK571, and not at all by 50 μM BSO (Fig. 5C).

Discussion

There is increasing evidence for an involvement of MRP in clinically occurring drug resistances (7-9) which points to the need for developing chemosensitizers also affecting this type of MDR. The mechanisms of the MRP-mediated drug efflux, though, are little understood, and very few reports (10-13) presently exist on compounds influencing a MRP associated MDR. One of the studies described the modulation of anthracycline accumulation in a variety of non-P-gp MDR cell lines including the cell line GLC4/ADR by the isoflavonoid genistein, which inhibits tyrosine kinases. There, MDR modulation was seen at rather high concentrations of genistein (200 μM) which were beyond the inherent cytotoxicity of the drug itself (12). Hence, MDR modulation by this drug cannot be examined in cell proliferation assays. Recently, we have shown that the MRP associated MDR of HL60/AR or GLC4/ADR cells is efficiently modulated by the selective bisindolylmaleimide PKC inhibitor GF 109203X (29). It remains to be elucidated, however, whether these aforementioned compounds might act via direct interaction with the MRP or otherwise. The chemosensitizing effects of BSO, on the other hand, are supposedly caused by a depletion of intracellular GSH pools. The latter could be effective either by a lowered protection of cells from radical species produced in the presence of anthracyclines and oxygen (16), or a lowered capacity of the cell for biotransformation of drugs via glutathione-S-transferases (19). However, the distinct reversal of resistance towards the anthracycline daunorubicin by BSO on HL60/AR cells reported in (17) was associated with increased cellular drug accumulation and retention suggesting that transport processes might be involved. This is in good agreement with our observation of a complete reversal of vincristine resistances exclusively in the MRP overexpressing cell lines by BSO, because no radical mediated cytotoxicity of vincristine is known so far, i.e. the reversal of vincristine resistance by BSO can be explained by a reduced intracellular supply of drug GSH conjugates for export via MRP. The relatively high inherent cytotoxicity and chemosensitizing efficacy of BSO on HL60/AR cells might be explained by their already lowered GSH level compared to the parental HL60 cells (17). A collateral sensitivity towards BSO was also described on the MRP overexpressing small cell lung cancer cell line H69/AR (5, 30), but no enhancement of adriamycin sensitivity by BSO could be detected there. This contrasts to our results found on GLC4/ADR cells. As outlined (19), little is known on biotransformation of anticancer drugs like vincristine or adriamycin in various types of tumor cells. Thus, our data obtained by applying BSO provide only circumstantial evidence for an MRP mediated efflux of vincristine or adriamycin as GSH conjugates. Nonetheless, the reports on competition of [^{3}H]LTC_4 photoaffinity labeling by MK571 on MRP together with the reports on inhibition of [^{3}H]LTC_4 transport into membrane vesicles prepared from MRP transfectants by MK571 (22, 23) gave strong evidence for MRP representing a conjugate transporter (which surely does not exclude that other types of substrates are transported by MRP as well). However, our results of the modulating activity of MK571 on the resistances of MRP overexpressing cells towards the two structurally

and mechanistically unrelated anticancer drugs vincristine and adriamycin further support this view. Considering the results presented in (22, 23) the assumption appears to be justified that MK 571 modulates MRP associated MDR via direct interaction with MRP. The effective final concentrations of MK571 are clearly higher in the experiments performed by us (≥ 10 μM) compared to the experiments using membrane vesicles prepared from MRP transfectants described in (23) where a K_i value of 0.6 μM for the inhibition of ATP-dependent LTC_4 transport by MK571 was measured. This discrepancy might be due to fact that in our work intact cells under regular cell culturing conditions, i.e. in the presence of 10% serum, are used for the assay.

It appears important to note that the compound MK571 represents the sodium salt of a lipophilic organic acid which contrasts to the structures of most chemosensitizers modulating a P-gp associated MDR. In accordance with this, no significant influence of MK571 on the drug resistance of the P-gp overexpressing HL60/DAU120 cells was seen. Therefore, our result not only support the published evidence (21-23) for a close relationhip - if not identity - of the 190 kDa MRP drug transporter with a conjugate transporter, but also present a new type of compound specifically reversing cellular resistance caused by overexpression of MRP.

We cannot explain the only partial reversion of adriamycin resistance by MK571. One might speculate, that in case of adriamycin some additional factors not susceptible to the modulator, e.g. an altered activity of topoisomerase II (31) or an increased expression of the LRP (lung cancer resistance associated protein) (32), could be responsible for this observation.

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References

1. Gottesman, M. M., and Pastan, I. (1993) Annu. Rev. Biochem. 62, 385-427.
2. Efferth, T., and Osieka, R. (1993) Tumordiagn. u. Ther. 14, 238-243.
3. Raderer, M., and Scheithauer, W. (1993) Cancer 72, 3553-3563.
4. McGrath, T., Latoud, C., Arnold, S. T., Safa, A. R., Felsted, R. L., and Center, M. S. (1989) Biochem. Pharmacol. 38, 3611-3619.
5. Cole, S. P. C., Bhardwaj, G., Gerlach, J. H., Mackie, J. E., Grant, K. E., Almquist, K. C., Stewart, A. J., Kurz, E. U., Duncan, A. M. V., and Deeley, R. G. (1992) Science 258, 1650-1654.
6. Grant, C. E., Valdimarsson, G., Hipfner, D. R., Almquist, K. C., Cole, S. P. C., and Deeley, R. G. (1994) Cancer Res. 54, 357-361.
7. Gekeler, V., Beck, J., Noller, A., Wilisch, A., Frese, G., Neumann, M., Handgretinger, R., Ehninger, G., Probst, H., and Niethammer, D. (1994) Ann. Hematol. 69, S19-S24.
8. Beck, J., Niethammer, D., and Gekeler, V. (1994) Cancer Lett. 86, 135-142.

9. Beck, J., Handgretinger, R., Dopfer, R., Klingebiel, T., Niethammer, D., and Gekeler, V. Br. J. Haematol. (in press).
10. Zijlstra, J. G., de Vries, E. G. E., and Mulder, N. H. (1987) Cancer Res. 47, 1780-1784.
11. Cole, S. P. C., Downes, H. F., and Slovak, M. L. (1989) Br. J. Cancer 59, 42-46.
12. Versantvoort, C. H. M., Schuurhuis, G. J., Pinedo, H. M., Eekman, C. A., Kuiper, C. M., Lankelma, J., and Broxterman, H. J. (1993) Br. J. Cancer 68, 939-946.
13. Barrand, M. A., Rhodes, T., Center, M. S., and Twentyman, P. R. (1993) Eur. J. Cancer 29A, 408-415.
14. Griffith, O. W., and Meister, A. (1979) J. Biol. Chem. 254, 7558-7560.
15. Hamilton, T. C., Winker, M. A., Louie, K. G., Batist, G., Behrens, B. C., Tsuruo, T., Grotzinger, K. R., McKoy, W. M., Young, R. C., and Ozols, R. F. (1985) Biochem. Pharmacol. 34, 2583-2586.
16. Dusre, L., Mimnaugh, E. G., Myers, C. E., and Sinha, B. K. (1989) Cancer Res. 49, 511-515.
17. Lutzky, J., Astor, M. B., Taub, R. N., Baker, M. A., Bhalla, K., Gervasoni, Jr., J. E., Rosado, M., Stewart, V., Krishna, S., and Hindenburg, A. A. (1989) Cancer Res. 49, 4120-4125.
18. Meijer, C., Mulder, N. H., Timmer-Bosscha, H., Peters, W. H. M., and de Vries, E. G. E. (1991) Int. J. Cancer 49, 582-586.
19. Tew, K. D. (1994) Cancer Res. 54, 4313-4320.
20. Schaub, T., Ishikawa, T., and Keppler, D. (1991) FEBS Lett. 279, 83-86.
21. Leier, I., Jedlitschky, G., Buchholz, U., and Keppler, D. (1994) Eur. J. Biochem. 220, 599-606.
22. Jedlitschky, G., Leier, I., Buchholz, U., Center, M., and Keppler, D. (1994) Cancer Res. 54, 4833-4836.
23. Leier, I., Jedlitschky, G., Buchholz, U., Cole, S. P. C., Deeley, R. D., and Keppler, D. (1994) J. Biol. Chem. 269, 27807-27810.
24. Bhalla, K., Hindenburg, A., and Taub, R. N., (1985) Cancer Res. 45, 3657-3662.
25. Zamboni, R., Belley, M., Champion, E., Charette, L., DeHaven, R., Frenette, R., Gauthier, J. Y., Jones, T. R., Leger, S., Masson, P. , McFarlane, C. S., Metters, K., Pong, S. S., Piechuta, H., Rokach, J., Thérien, M., Williams, H. W. R., and Young, R. N. (1992) J. Med. Chem. 35, 3832-3844.
26. Wilisch, A., Noller, A., Handgretinger, R., Weger, S., Nüssler, V., Niethammer, D., Probst, H., and Gekeler, V. (1993) Cancer Lett. 69, 139-148.
27. Tso, J. Y., Sun, X. H., Kao, T., Reece, K. S., and Wu, R. (1985) Nucleic Acids Res. 13, 2485-2503.
28. Mosmann, T. J. (1983) Immunol. Methods 65, 55-63.
29. Gekeler, V., Boer, R., Ise, W., Sanders, K. H., Schächtele, C., and Beck, J. (1995) Biochem. Biophys. Res. Commun. (in press).
30. Cole, S. P. C., Downes, H. F., Mirski, S. E. L., and Clements, D. J. (1990) Mol. Pharmacol. 37, 192-197.
31. De Jong, S., Zijlstra, J. G., de Vries, E. G. E., and Mulder, N. H. (1990) Cancer Res. 50, 304-309.
32. Scheper, R. J., Broxterman, H. J., Scheffer, G. L., Kaaijk, P., Dalton, W. S., van Heijningen, T. H. M., van Kalken, C. K., Slovak, M. L., de Vries, E. G. E., van der Valk, P., Meijer, C. J. M., and Pinedo, H. M. (1993) Cancer Res. 53, 1475-1479.