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THE SPECIFIC BISINDOLYLMALEIMIDE PKC-INHIBITOR GF 109203X EFFICIENTLY MODULATES MRP-ASSOCIATED MULTIPLE DRUG RESISTANCE

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Summary. The newly identified drug transporter MRP is functionally linked to a multiple drug resistance independent from P-glycoprotein. Resistance modifiers for this type of MDR are rare at present. We analyzed the modulating effect of the highly selective bisindolylmaleimide PKC inhibitor GF 109203X on the MRP overexpressing human MDR sublines HL60/AR and GLC4/ADR. Applying a 72 hour MTT-assay we demonstrate a complete reversal of the vincristine resistance of HL60/AR cells. Adriamycin resistance of HL60/AR, or vincristine resistance of GLC4/ADR were partially reversed. Furthermore, rhodamine 123 efflux from HL60/AR was strongly modulated by GF 109203X. Since the PKC inhibitor did not significantly influence MRP gene expression at the mRNA level which was examined by cDNA-PCR, our results suggest either a direct interaction of the compound with MRP or/and an indirect influence on MRP activity via altering the phosphorylation status of the transporter.

The ABC (=ATP binding cassette) transmembrane protein MRP (multidrug resistance associated protein) was recently identified (1, 2) as a cause of a MDR (multiple drug resistance) independent of the P-gp (mdr1/P-glycoprotein). The MRP accordingly represents a phosphoprotein like P-gp (3). In fact, some influence of PKC (protein kinase C) on a P-gp associated MDR either via phosphorylation of P-gp or/and modulation of mdr1 gene expression is indicated by the work of several groups, and inhibition of PKC is discussed as a new approach for overcoming MDR in cancer chemotherapy (4-7). Therefore, it seems reasonable to ask whether a MRP associated MDR could be influenced by a PKC inhibitor. The bisindolylmaleimide GF 109203X which is identical with Gö 6850 (Fig. 1) shows highly selective inhibition of many PKC isozymes in vitro (IC_{50}: 30-200 nM) except PKC ζ (IC_{50}: 6 μM), while other kinases are affected only at distinctly higher concentrations (8-11), e.g. PKA (cAMP dependent protein kinase; IC_{50}: 33 μM), G-kinase (cGMP dependent kinase; IC_{50}: 4.6 μM), or tyrosine kinases (IC_{50}: 94 μM). This compound is therefore suited to address the issue. For investigation of the

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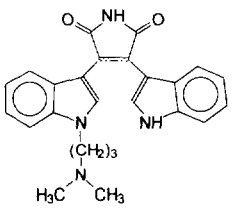

Bisindolylmaleimide
(GF 109203X = Gö 6850)

Figure 1. Structure of the bisindolylmaleimide GF 109203X.

biological effects on the MRP overexpressing cell lines HL60/AR (12) and GLC4/ADR (13), we used a 72 h tetrazolium based MTT-assay. As PKC is discussed to represent a part of a stress response activating e.g. the mdr1 gene, and PKC inhibitors thus might be able to attenuate the activation of drug resistance associated genes (14, 15), we also examined the effect of the compound GF 109203X on relative mrp mRNA levels by cDNA-PCR (complementary DNA polymerase chain reaction).

The clinical relevance of MRP as a mechanism to protect tumor cells from chemotherapy is unknown at present. Nonetheless, we recently reported on combined increases of mdr1/P-gp and MRP gene expression in different types of leukemias not responding anymore to chemotherapy (16-18). Thus, the identification of chemosensitizers modulating a MRP associated MDR might be valuable for developing new strategies for overcoming MDR of tumor cells during cancer chemotherapy.

Materials and Methods

Cell lines. The parental, promyelocytic human leukemia cell line HL60 (ATCC CCL 240), and its non-P-gp MDR subline HL60/AR (12) were cultured in RPMI1640/10% FCS medium, the latter additionally in the presence of 100 nM daunomycin. The human small cell lung cancer cell line GLC4, and its non-P-gp MDR subline GLC4/ADR (13) were cultured under the same conditions, the MDR subline was maintained in the presence of 680 ng/ml adriamycin.

Drugs. GF 109203X was purchased from Calbiochem (Bad Soden/Germany). Vincristine-sulfate (VCR), doxorubicin-HCl (adriamycin, ADR), daunomycin-HCl, and rhodamine 123 were purchased from Sigma (Deisenhofen/Germany).

Drug sensitivity testing. Prior to analysis the MDR sublines were cultivated without the drug used for selection for about 1 to 2 weeks. The tetrazolium based colorimetric MTT-assay (19) was applied for determination of drug sensitivities of the cell lines in the absence or presence of modulators. Therefore, cell aliquots 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.

Rhodamine 123 efflux. The assay was performed as reported recently (20), all incubations, however, were done here in the presence of complete culture medium including 10% fetal calf serum. After washing with medium the samples were incubated in the presence of rhodamine 123 (800 ng/ml) without or with GF 109203X for 60 min at 37 °C. The cells were washed and resuspended in dye-free medium without or with modulators, and incubation at 37 °C was continued for the times

indicated. Cells were analyzed on an Epics Profile II FACS (Coulter, Krefeld/Germany). The excitation wavelength was 488 nm, and the cell associated rhodamine 123 fluorescence was measured at 520 nm.

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 (21). The conditions for PCR were modified according to (17). The following amplimers were used for 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; 23 cycles, 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 (1, 22). 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.

Results and Discussion

The non-P-gp MDR cell lines used in the present work showed distinct relative resistances to anticancer drugs (12, 13) which were approved applying MTT-assays. Calculating the ratios of IC_{50} values of the MDR sublines and the parental cell lines, we measured resistances towards vincristine of 38-fold (HL60/AR), and 23-fold (GLC4/ADR), towards adriamycin 99-fold (HL60/AR), and 683-fold (GLC4/ADR), respectively. The bisindolylmaleimide GF 109203X inhibited cell growth showing IC_{50} values of about 14 μM (HL60 and HL60/AR), or 7 μM (GLC4 and GLC4/ADR), respectively. As the drug retains an almost unaltered inhibitory quality in a cellular assay measuring the c-fos promoter activity (23), we have no reason to suspect a significant inactivation of the agent during cell culturing. Therefore, we conclude that a specific inhibition of the PKC isozymes affected by GF 109203X at submicromolar concentrations in vitro, i.e. PKC α, β1, β2, γ, δ, and ϵ (8, 9) seems not to translate in a corresponding inhibition of cellular proliferation of the cell lines used in the present work under the chosen culturing conditions, e.g. 10% fetal calf serum. The small cell lung cancer cells, however, were clearly more sensitive to the agent.

The overexpression of the MRP gene in the MDR subline HL60/AR, and the influence of the compound GF109203X thereon is shown in Fig. 2. The cDNA-PCR was performed by increasing the number of PCR cycles. The signal intensities were calculated after normalization onto the signal intensities obtained by the amplimers for gapdh where 23 PCR cycles were applied throughout which was proven to be in the exponential range of PCR yield. Incubation of the HL60/AR cells for 24 hours without or with a final concentration of 7.5 μM GF 109203X was performed under standard conditions at an initial cell densitiy of $1x10^6$ cells/ml. Thereafter, cell counting did not reveal any effect on cellular proliferation or vitality of the cells, which is consistent with the data obtained by MTT-assays. Furthermore, the cDNA-PCR gene expression analysis showed that the specific PKC inhibitor did not alter the expression of the MRP gene at the mRNA level significantly. The analysis of material prepared from

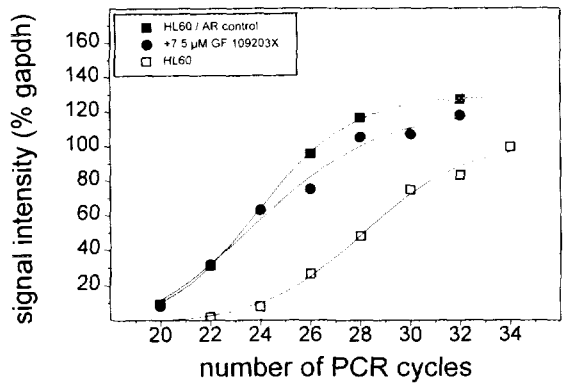

Figure 2. Kinetics of MRP gene expression analysis by cDNA-PCR for parental HL60 cells, and HL60/AR cells untreated or treated with 7.5 µM GF 109203X for 24 hours.

GLC4/ADR cells revealed a very similar overexpression of the MRP gene as found for HL60/AR cells (data not shown). The virtual absence of any significant mdr1 gene expression in HL60/AR and GLC4/ADR cells was approved by cDNA-PCR (data not shown).

Nonetheless, applying 72 h MTT-assays a distinct reversal of resistances was seen using final drug concentrations between 1 µM and 20 µM. Thus, starting with 1 µM the vincristine resistance of HL60/AR was decreased in a dose dependent manner, and the sensitivity of the parental HL60 cell line was approached at 8 µM of GF 109203X showing no antiproliferative quality at this concentration by itself. By increasing the drug doses further, the combined antiproliferative and MDR modulatory action of the compound become evident (Fig. 3A). The modulation of the adriamycin resistance of HL60/AR by the bisindolylmaleimide is shown in Fig. 3B. A pronounced effect was seen, a complete reversal was not achievable, though. Fig. 3C demonstrates that the compound only slightly affects the vincristine sensitivity of the parental HL60 cell line. The marginal influence might be due to a residual expression of the MRP gene there (see Fig. 2).

As we observed a clearly enhanced efflux of the fluorescent dye rhodamine 123 from the MRP overexpressing HL60/AR cells compared to parental HL60 cells (Fig. 4), the modulation of this efflux by the bisindolylmaleimide was investigated. At a final concentration of 10 µM a strong inhibition of the dye efflux was seen in very good aggreement with the results presented above (Fig. 4). This demonstrates that the compound GF 109203X actually affects a transport function most likely associated with MRP.

The influence of the bisindolylmaleimide on the vincristine resistance of GLC4/ADR cells is shown in Fig. 5. Due to the higher sensitivity of these cell towards the

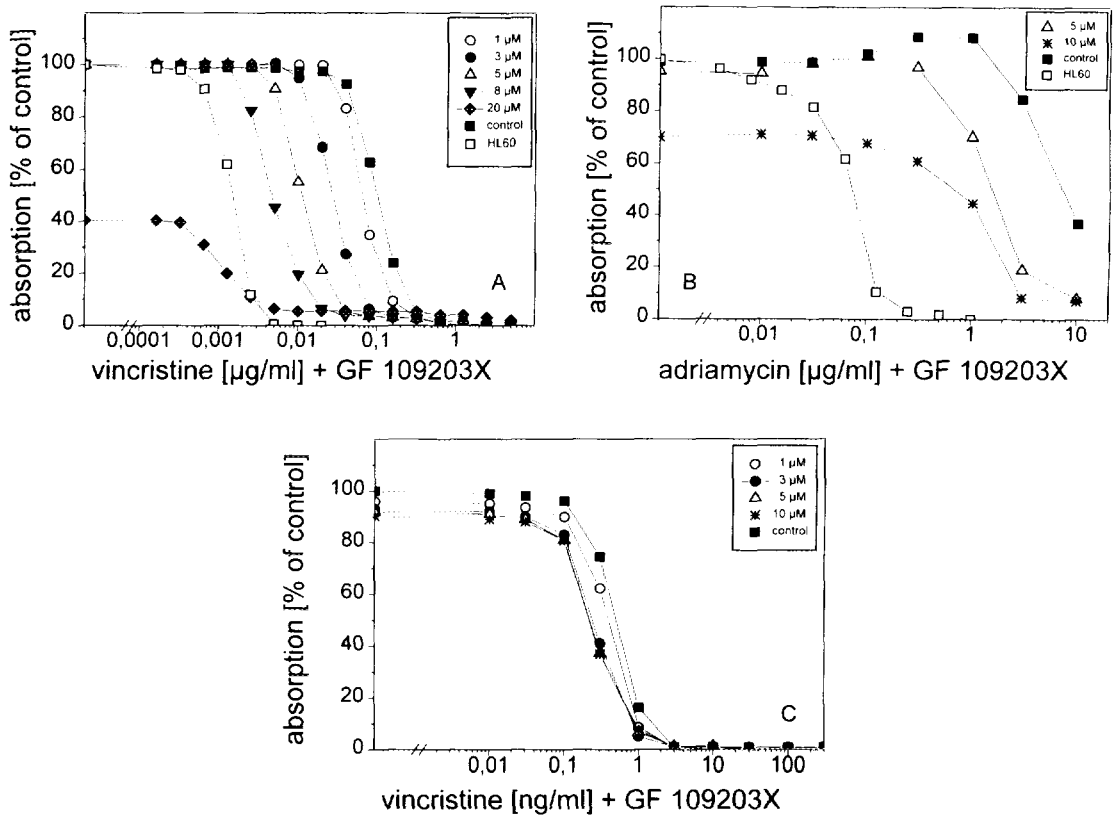

Figure 3. Dose-dependent reversion by GF 109203X of (A) vincristine, or (B) adriamycin resistances of the MRP overexpressing MDR subline HL60/AR, and (C) the influence GF 109203X on vincristine sensitivity of parental HL60 cells.

bisindolylmaleimide itself, the resistance modulating activity appears to be overlapped thereby.

Modulators influencing a MRP associated MDR are rare at present. Some activity of verapamil, cyclosporin A and its nonimmunosuppressive derivative SDZ PSC 833 was recently described using the multidrug-resistant, human large cell lung cancer cell line COR-L23/R which obviously also overexpresses MRP (24). Another study 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 (25).

The bisindolylmaleimide GF 109203X is very specific for PKC and, allegedly, does not influence the activity of tyrosine kinases in the micromolar concentration range whatsoever (8-10). Thus, our results disclose a new type of chemosensitizer, acting

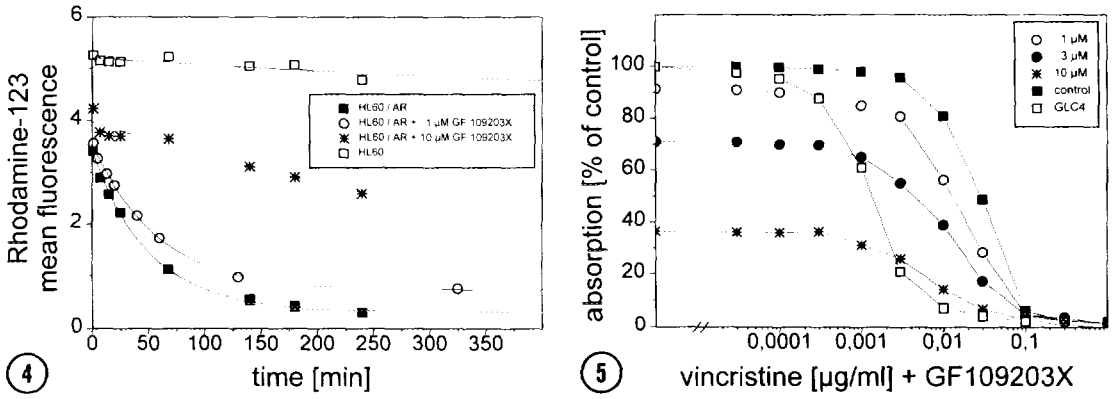

Figure 4. Efflux of rhodamine 123 from HL60 and HL60/AR cells, respectively, and modulation of rhodamine 123 efflux from HL60/AR cells by GF 109203X.

Figure 5. Dose-dependent reversion by GF 109203X of vincristine resistance of the MRP overexpressing MDR subline GLC4/ADR.

already at concentrations not cytotoxic to the cells by itself, for a MRP associated MDR. This appears to be important in view of the accumulating evidence for a MRP induced MDR, or a multifactorial emergence of MDR not only after selection of drug-resistant cell lines in vitro, but also in the clinics (16-18). The mechanism(s) targeted by the bisindolylmaleimide GF 109203X are unclear at present. We and others have shown that the compound is an effective PKC inhibitor in vitro on PKC preparations and in cellular systems in the submicromolar concentration range (8-10, 24). On the other hand, we demonstrate in the present work that the drug has no direct influence on MRP gene expression under the conditions applied (Fig. 2). Thus, either a PKC dependent function could be disturbed by GF 109203X, i.e. phosphorylation of MRP, or/and the compound interacts directly with the drug transporter. Because of the rather high concentrations of GF 109203X necessary to modulate the resistances of the non-P-gp MDR cells, the assumption of a direct interaction with the transporter appears to be reasonable. According to published data (8) the bisindolylmaleimide inhibits PKC competitively with ATP. This might suggest that the ATP-binding site(s) of MRP are affected here as well. Furthermore, our result do not indicate any influence of many PKC isozymes on cellular proliferation under the chosen conditions. The reasons for the two-fold higher sensitivity of the small cell lung cancer cell lines towards GF 109203X are unclear at present. We also cannot explain the only partial reversion of adriamycin resistance by this agent. One might speculate, that in case of adriamycin some other factors not susceptible to the modulator, e.g. an altered activity of topoisomerase II (26) or an increased expression of the LRP (lung cancer resistance associated protein), are responsible for this observation (27).

Acknowledgments

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References

1. 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.
2. 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.
3. Deeley, R. G., Grant, C. E., Almquist, K. C., Hipfner, D. R., Valdimarsson, G., Loe, D. W., Callen, D. F., Kuss, B., and Cole, S. P.C. (1994) Proc. Am. Assoc. Cancer Res. 35, 698-699.
4. Ahmad, S., and Glazer, R. I. (1993) Mol. Pharmacol. 43, 858-862.
5. Chambers, T. C., Pohl, J., Raynor, R. L., and J. F. Kuo. (1993) J. Biol.Chem. 268, 4592-4595.
6. Bates, S. E., Lee, J. S., Dickstein, B., Spolyar, M., and Fojo, A. T. (1993) Biochemistry 32, 9156-9164.
7. Grunicke, H., Hofmann, J., Utz, I., and Überall, F. (1994) Ann. Hematol. 69, S1-S6.
8. Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., Loriolle, F., Duhamel, L., Charon, D., and Kirilovsky, J. (1991) J. Biol. Chem. 266, 15771-15781.
9. Martiny-Baron, G., Kazanietz, M. G., Mischak, H., Blumberg, P. M., Kochs, G Hug, H., Marmé, D., and Schächtele, C. (1993) J. Biol. Chem. 268, 9194-9197.
10. Hartenstein, J. H., Aranda, J., Barth, H., Kleinschroth, J., Reck, R., Rudolph, C., Trostmann, U., and Schächtele, C. (1993). In: Perspectives in Medicinal Chemistry (B. Testa, E. Kyburz, W. Fuhrer, R. Giger, Eds.), pp 99-118, Verlag Helvetica Chimica Acta, Basel, Switzerland.
11. Qatsha, K. A., Rudolph, C., Marmé, D., Schächtele, C., and May, W. S. (1993) Proc. Natl. Acad. Sci. USA 90, 4674-4678.
12. Bhalla, K., Hindenburg, A., and Taub, R. N., (1985) Cancer Res. 45, 3657-3662.
13. Zijlstra, J. G., de Vries, E. G. E., and Mulder, N. H. (1987) Cancer Res. 47, 1780-1784.
14. Chaudary, P. M., and Roninson, I. B. (1992) Oncol. Res. 4, 281-290.
15. Chaudary, P. M., and Roninson, I. B. (1993) J. Natl. Cancer Inst. 85, 632-639.
16. 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.
17. Beck, J., Niethammer, D., and Gekeler, V. (1994) Cancer Lett. (in press).
18. Beck, J., Handgretinger, R., Dopfer, R., Klingebiel, T., Niethammer, D., and Gekeler, V. Br. J. Haematol. (in press).
19. Mosmann, T. J. (1985) Immunol. Methods 65, 55-63.
20. Boer, R., Haas, S., and Schödl, A. (1994) Eur. J. Cancer 30A, 1117-1123.
21. Wilisch, A., Noller, A., Handgretinger, R., Weger, , Nüssler, V., Niethammer, D., Probst, H., and Gekeler, V. (1993) Cancer Lett. 69, 139-148.
22. Tso, J. Y., Sun, X. H., Kao, T., Reece, K. S., and Wu, R. (1985) Nucleic Acids Res. 13, 2485-2503.

23. Gekeler, V., Beck, J., Boer, R., Giel, M., Ise, W., Überall, F., Borchers, C., Sanders, K. H., Schächtele, C., Klemm, K., and Grunicke, H. (1994) Proc. Am. Assoc. Cancer Res. 35, 357.
24. Barrand, M. A., Rhodes, T., Center, M. S., and Twentyman, P. R. (1993) Eur. J. Cancer 29A, 408-415.
25. 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.
26. De Jong, S., Zijlstra, J. G., de Vries, E.G.E., and Mulder, N.H. (1990) Cancer Res. 50, 304-309.
27. 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. L. M., and Pinedo,. H. M. (1993) Cancer Res. 53, 1475-1479.