Cancer Chemother Pharmacol (1995) 35: 345–348

SHORT COMMUNICATION

Winette T. A. van der Graaf · Nanno H. Mulder
Coby Meijer · Elisabeth G. E. de Vries

The role of methoxymorpholino anthracycline and cyanomorpholino anthracycline in a sensitive small-cell lung-cancer cell line and its multidrug-resistant but *P*-glycoprotein-negative and cisplatin-resistant counterparts

Received: 21 July 1993 / Accepted: 22 July 1994

Abstract The cytotoxic action of two morpholino anthracyclines, methoxymorpholino anthracycline (MRA-MT, FCE 23762) and cyanomorpholino anthracycline (MRA-CN), was compared with the cytotoxicity of doxorubicin (DOX), the topoisomerase II inhibitor etoposide (VP-16), the topoisomerase I inhibitor camptothecin, methotrexate, and cisplatin in GLC4, a human small-cell lung-cancer cell line, in GLC4-Adr, its P-glycoprotein (Pgp)-negative, multidrug-resistant (MDR; 100-fold DOX-resistant) subline with overexpression of the MDR-associated protein (MRP) and a lowered topoisomerase II activity, and in GLC4-CDDP, its cisplatin-resistant subline. GLC4-Adr was about 2-fold cross-resistant for the morpholino anthracyclines and GLC4-CDDP was, relative to GLC4, more resistant for the morpholino anthracyclines than for DOX. Overall, MRA-CN was about 2.5-fold more cytotoxic than MRA-MT. The cytotoxicity profile of the morpholino anthracyclines in these cell lines mimicked that of camptothecin.

Key words Morpholino anthracyclines · MRP
Cisplatin resistance

Introduction

The development of multidrug resistance (MDR) is one of the major obstacles in successful chemotherapeutic treatment of cancer patients. One class of antitumor drugs with the widest spectrum of activity in human cancers comprises the anthracyclines, which, however, are involved in MDR [1]. For doxorubicin (DOX), one of the most frequently used drugs of this group of anthracyclines, several mechanisms contribute to its cytotoxic action. Cellular mechanisms of action include intercalation of DNA; the formation of DNA breaks, possibly due to the generation of free radicals; and the capacity to turn DOX-topoisomerase complexes into cellular poisons [2, 3]. Because of its toxicity, especially cardiac toxicity, and the appearance of drug resistance, new anthracycline analogs have been synthesized that are less toxic, more potent, and non-cross-resistant with DOX. One group of these analogs consists of compounds in which a morpholino ring incorporating the amino nitrogen of the daunosamine unit has been constructed. Acton et al. [4] synthesized a series of morpholino (e.g., MRA) and cyanomorpholino (e.g., MRA-CN) analogs of DOX. These morpholinyl-substituted anthracyclines have several properties in common that distinguish them from the parental anthracyclines. They are highly lipophilic, which facilitates rapid diffusion through the cell membrane [4, 5]. In contrast to DOX, MRA and MRA-CN are not cardiotoxic at effective antitumor doses [4, 6] and are not cross-resistant in DOX-resistant P-glycoprotein (Pgp)-positive and -negative cell lines [6–9]. Apart from its efficacy in Pgp-positive cell lines resistant to DOX, MRA-MT has also been proven effective in CEM/VM-1, a cell line with altered topoisomerase II, and in cell lines resistant to cisplatin and melphalan [10–13].

The working mechanisms of MRA and MRA-CN include preferential inhibition of ribosomal gene transcription [14] as well as topoisomerase I-mediated DNA cleavage [15]. This mechanism of cytotoxicity is probably different from that of DOX, which acts on topoisomerase II. Whereas MRA binds to DNA by intercalation and causes DNA strand breaks, MRA-CN produces DNA-DNA interstrand cross-links [15, 16]. It has been demonstrated that this interstrand DNA cross-link formation induced by MRA-CN, which takes place very rapidly, is preceded by the binding of drug to single-stranded DNA [17]. The marked difference in cytotoxicity and DNA-binding affinity observed between MRA and MRA-CN suggests a major role for the cyano substituent in the action of MRA-CN [18].

W. T. A. van der Graaf (✉) · N. H. Mulder · C. Meijer · E. G. E. de Vries
Department of Internal Medicine, Division of Medical Oncology, University Hospital, Oostersingel 59, 9713 EZ Groningen, The Netherlands

Table 1 ID_{50} values after 1 h incubation as determined in the MTA. Results are expressed as mean values (± SD) for 2–4 experiments performed in quadruplicate

	Cell lines		
	GLC4	GLC4-Adr	GLC4-CDDP
DOX (μ*M*)	0.33 ±0.07	30.1 ±8.0	0.17 ±0.06
MRA-MT (μ*M*)	0.0079±0.0017	0.020±0.005	0.030±0.009
MRA-CN (μ*M*)	0.0045±0.0018	0.0052±0.0020	0.0088±0.0009

Table 2 ID_{50} values after continuous incubation as determined in the MTA. Results are expressed as mean values (± SD) for 2–7 experiments performed in quadruplicate

	Cell lines		
	GLC4	GLC4-Adr	GLC4-CDDP
DOX (n*M*)	32.5 ±2.1	3,732 ±336	42.3 ± 1.0
VP-16 (n*M*)	0.16±0.03	10.2 ± 2.4	0.11± 0.02
Camptothecin (n*M*)	6.1 ±2.5	7.6 ± 0.9	30.8 ±14.4
Methotrexate (μ*M*)	0.05±0.03	0.14± 0.04	0.08± 0.02
Cisplatin (μ*M*)	0.90±0.07	2.2 ± 0.2	11.9 ± 2.2
MRA-CN (n*M*)	0.59±0.16	1.4 ± 0.4	2.1 ± 1.4

In the present study the role of MRA-CN and MRA-MT in cell lines with well-defined, different patterns of resistance, namely, non-Pgp MDR and cisplatin resistance, was tested and compared with the cytotoxicity of these compounds in a sensitive cell line.

Materials and methods

GLC4 is a Pgp-negative human small-cell carcinoma cell line [19] and GLC4-Adr is the DOX-resistant subline of GLC4. It shows an atypical MDR phenotype with resistance to DOX, vincristine, VP-16, and m-AMSA without *mdr*-1 gene amplification or Pgp expression [20]. In GLC4-Adr, a membrane efflux pump different from Pgp and overexpression of a mainly cytoplasmic 110-kDa protein detectable with the monoclonal antibody LRP-56 as well as overexpression of the new putative membrane transporter gene MRP were demonstrated [21–23]. GLC4-CDDP is the cisplatin-resistant subline of GLC4 with a 13.2-fold resistance to cisplatin due to increased glutathione (GSH), unchanged glutathione S-transferase (GST), decreased DNA platination, and increased repair of platinum adducts [24, 25]. Topoisomerase II activity proved to be 100% in GLC4, 35% in GLC4-Adr, and 130% in GLC4-CDDP [26]. Topoisomerase I activity did not differ among these cell lines [20]. GSH levels are 2.5-fold higher in GLC4-CDDP and 2.1-fold higher in GLC4-Adr as compared with GLC4; GST activity is equal in GLC4 and GLC4-CDDP but is 1.7-fold higher in GLC4-Adr [25, 27]. The doubling times of these cell lines are as follows: GLC4, 16.5 h; GLC4-Adr, 21.8 h; and GLC4-CDDP, 28.0 h [25, 28]. All cell lines were cultured in RPMI 1640 medium and 10% fetal calf serum (FCS) at 37 °C in a humidified atmosphere containing 5% CO_2.

A drug-sensitivity assay was performed with the microculture tetrazolium assay (MTA) as described previously [28]. To assure linearity the following numbers of cells per well (0.1 ml) were incubated: GLC4, 5,000; GLC4-Adr, 12,500; and GLC4-CDDP, 15,000. Cells were incubated with chemotherapeutic drugs either continuously for 4 days or for 1 h. When incubated for 1 h, the cells were washed. All assays were performed two to seven times in quadruplicate. The results are expressed as the mean (± SD) doses required to inhibit the growth of each cell line by 50% (ID_{50} values).

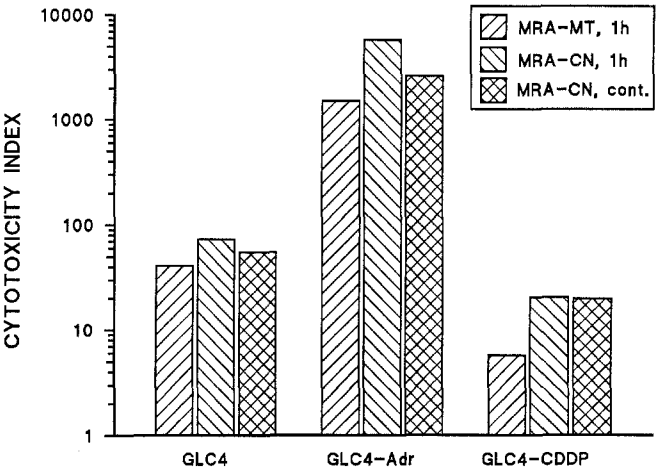

Fig. 1 Cytotoxicity index (ratio of the ID_{50} for DOX versus the ID_{50} for morpholino anthracyclines as determined in the MTA) obtained for each cell line

To measure the effect of buthionine sulfoximine (BSO) pretreatment on MRA-CN-induced cytotoxicity, GLC4, GLC4-Adr, and GLC4-CDDP cells were cultured for 48, 24, and 48 h, respectively, in the presence of 50 μ*M* BSO without growth delay or loss of viability. Subsequently, MRA-CN-induced cytotoxicity (continuous incubation) was measured in the MTA (n = 3–4 experiments performed in quadruplicate).

Results

Tables 1 and 2 show the results of the 1-h and continuous incubations, respectively, in the MTA. Both morpholino anthracyclines are much more potent than DOX in these cell lines. The cytotoxicity of the morpholinyl derivates is remarkable in the highly DOX-resistant GLC4-Adr line. In contrast, GLC4-CDDP is relatively more sensitive to DOX but less sensitive to MRA-MT and MRA-CN than is GLC4. This comparison of the cytotoxicity sensitivity of the different cell lines for DOX, MRA-MT, and MRA-CN is expressed in Fig. 1. In this figure the cytotoxicity index is shown, which represents the ratio of the ID_{50} determined for DOX either after a 1-h incubation or after continuous incubation in a certain cell line versus the ID_{50} found for MRA-MT or MRA-CN. This demonstrates the potency of MRA-MT and MRA-CN with respect to DOX but also gives an impression about the mutual efficacy of the two morpholino anthracyclines and about the possibly different results of short versus continuous incubation. In all cell lines the cytotoxic action of the morpholino compounds surpassed the cytotoxicity of DOX. For both MRA-MT and MRA-CN the cytotoxicity indices were lowest in GLC4-CDDP and highest in GLC4-Adr. In the 1-h incubations, MRA-CN was 1.8-fold more active than MRA-MT in GLC4, 3.4-fold more active in GLC4-CDDP, and 3.8-fold more active in GLC4-Adr. In GLC4 and GLC4-Adr, 1-h incubations of MRA-CN seemed more effective than continuous incubations, whereas in GLC4-CDDP there was no difference.

Table 3 Cross-resistance factors[a] of GLC4-Adr and GLC4-CDDP versus GLC4 for DOX, MRA-MT, and MRA-CN

	Cell lines	
	GLC4-Adr	GLC4-CDDP
DOX	91	0.5
MRA-MT	2.5	3.8
MRA-CN	1.2	2.0

[a] At the ID_{50} as determined in the MTA (1 h incubation)

Table 4 Cross-resistance factors[a] of GLC4-Adr and GLC4-CDDP versus GLC4 for DOX, VP-16, camptothecin, methotrexate, cisplatin and MRA-CN

	Cell lines	
	GLC4-Adr	GLC4-CDDP
DOX	115	1.3
VP-16	64	0.7
Camptothecin	1.2	5.0
Methotrexate	2.8	1.6
Cisplatin	2.3	13.2
MRA-CN	2.4	3.6

[a] At the ID_{50} as determined in the MTA (continuous incubation)

The results of the MTA also led to cross-resistance factors, which are shown in Tables 3 and 4. The cross-resistance factors were calculated from the ratio of the ID_{50} determined for a certain chemotherapeutic drug in GLC-Adr and GLC4-CDDP, respectively, versus the ID_{50} in GLC4. The cross-resistance for DOX in GLC4-Adr was remarkably reduced for both morpholino compounds. This was also the case for camptothecin. GLC4-Adr showed some cross-resistance for methotrexate and cisplatin but was highly cross-resistant for VP-16. GLC4-CDDP was relatively insensitive to the morpholino anthracyclines as well as to camptothecin but was sensitive to DOX, VP-16, and methotrexate.

The effects of pretreatment with BSO on MRA-CN-induced cytotoxicity, expressed as dose-modifying factors at the ID_{50} as determined after continuous incubation in the MTA, were as follows: GLC4, 0.90 ± 0.06; GLC4-Adr, 1.04 ± 0.02; and GLC4-CDDP, 1.18 ± 0.26. Thus, BSO slightly increased MRA-CN-induced cytotoxicity in GLC4-CDDP cells.

Discussion

In our panel of cell lines, MRA-MT and MRA-CN are both very active chemotherapeutic drugs as compared with DOX. Both drugs are most active in GLC4-Adr, the cell line that is about 100-fold resistant to DOX. In this cell line, MRA-CN proved to be 5,790- and 2,590-fold more active than DOX after 1 h and continuous exposure, respectively. This is an interesting observation because of the remarkable properties of GLC4-Adr. Hence, in the MDR H69AR cell line, which also overexpresses MRP [29], Cole [30] reported a relative lack of potency for MRA-CN. However, in this cell line, no DOX-accumulation deficit exists [29]. From our observations the conclusion might be drawn that MRP, just as Pgp-mediated MDR, does not seem to be involved in the sensitivity to MRA-CN. Moreover, these morpholino anthracyclines circumvent altered topoisomerase II activity, as was demonstrated for their cytotoxic activity in the GLC4-Adr cell line. The relative lack of activity found for MRA-MT and, to a lesser degree, also for MRA-CN in GLC4-CDDP cells has not previously been described in a cisplatin-resistant cell line. Ripamonti et al. [13] reported an equivalent efficacy for MRA-MT in the wild-type and in the cisplatin-resistant murine leukemia cell line L1210. A 4-fold MRA-CN-resistant ES-2R cell line, however, also shows 7-fold cross-resistance to cisplatin [31]. The role of an enhanced amount of detoxifying activity, as has been reported for ES-2R as well as for GLC4-CDDP, might play a role in this cross-resistance between MRA-CN and cisplatin [31–33]. This was also partly suggested by the results of our experiments with BSO modulation on MRA-CN-induced cytotoxicity in GLC4-CDDP cells.

Concerning an elucidation of the working mechanisms of MRA-MT and MRA-CN in our cell lines, it is interesting that the cytotoxicity profiles of these drugs in the GLC4 cell lines mimic those of camptothecin and differ markedly from those of VP-16. This confirms earlier observations in which the cytostatic action of morpholino and cyano-morpholino doxorubicin was attributed to DNA topoisomerase I-induced cleavage and not to topoisomerase II-induced cleavage [15]. This finding has to be confirmed in other cell lines.

In our cell lines the activity of MRA-CN was about 2.5-fold that of MRA-MT. Previously, MRA-MT was reported to be 3- to 15-fold more potent than DOX in various cell lines, whereas MRA-CN was 100- to 1,000-fold more potent than DOX [9, 10]. We found an increase in the cytotoxicity of MRA-MT versus DOX that varied between 6- and 1,500-fold. The reason why our observations differ from the previous reports is not clear, as a modest difference in potency between MRA-MT and MRA-CN was observed in all our tested cell lines. The duration of incubation of MRA-CN made no uniform difference in its cytotoxic activity. Althoug the cross-link formation induced by MRA-CN takes place much faster than that caused by, e.g., cisplatin, this apparently has no effect on its final cytotoxic potential. Because of the generally promising cytostatic potency of the morpholino anthracyclines, the results of clinical studies, of which only a few have been reported to date, are awaited with great interest [34, 35].

In conclusion, MRA-MT and MRA-CN are highly potent chemotherapeutic drugs in a DOX-resistant cell line with overexpression of MRP and lowered topoisomerase II activity. Cross-resistance for the morpholino anthracyclines was found in a cisplatin-resistant cell line, suggesting a role for detoxifying systems such as GSH and GST. Topoisomerase I-mediated cytotoxicity is suggested because of the comparable cytotoxicity of the morpholino anthracyclines and camptothecin in the small-cell lung-cancer cell lines. MRA-CN is ca. 2.5-fold more active

than MRA-MT, whereas the duration of incubation does not play a uniform role in its cytotoxic potency.

Acknowledgement This study was supported by grant GUKC 91-12 from the Dutch Cancer Society.

References

1. Bradley G, Juranka PF, Ling V (1988) Mechanism of multidrug resistance. Biochim Biophys Acta 948: 87
2. De Vries EGE, Zijlstra JG (1990) Morpholinyl anthracyclines: option for reversal of anthracycline resistance. Eur J Cancer 26: 659
3. Tewey KM, Rowe TC, Yang L, Halligan BD, Liu LF (1984) Adriamycin-induced DNA damage mediated by mammalian topoisomerase II. Science 226: 466
4. Acton EM, Tong GL, Mosher CW, Wolgemuth RL (1984) Intensely potent morpholinyl anthracyclines. J Med Chem 27: 638
5. Streeter DG, Johl JS, Gordon GR, Peters JH (1986) Uptake and retention of morpholinyl anthracyclines by adriamycin-sensitive and -resistant P388 cells. Cancer Chemother Pharmacol 16: 247
6. Sikic BI, Ehsan MN, Harker WG, Friend NF, Brown BW, Newman RA, Hacker MP, Acton EM (1985) Dissociation of antitumor potency from anthracycline cardiotoxicity in a doxorubicin analog. Science 228: 1544
7. Coley HM, Twentyman PR, Workman P (1989) Identification of anthracyclines and related agents that retain preferential activity over adriamycin in multidrug-resistant cell lines, and further resistance modification by verapamil and cyclosporin A. Cancer Chemother Pharmacol 24: 284
8. Coley HM, Workman P, Twentyman PR (1991) Retention of activity by selected anthracyclines in a multidrug resistant human large cell lung carcinoma line without P-glycoprotein hyperexpression. Br J Cancer 63: 351
9. Streeter DG, Taylor DL, Acton EM, Peters JH (1985) Comparative cytotoxicities of various morpholinyl anthracyclines. Cancer Chemother Pharmacol 14: 160
10. Grandi M, Pezzoni G, Ballinari D, Capolongo L, Suarato A, Bargiotti A, Faiardi D, Spreafico F (1990) Novel anthracycline analogs. Cancer Treat Rev 17: 133
11. Grandi M, Mariani M, Ballinari D, Pezzoni G, Suarato A, Sreafico F, Chen M, Danks MK, Beck WT (1990) Lack of cross-resistance to certain anthracycline analogs in human leukemic multidrug resistant cells expressing either P-glycoprotein or altered DNA topoisomerase II. Proc Am Assoc Cancer Res 31: 2118
12. Lau DHM, Lewis AD, Durán GE, Sikic BI (1991) The cellular and biochemical pharmacology of the methoxymorpholino derivative of doxorubicin, FCE 23762. Proc Am Assoc Cancer Res 32: 1970
13. Ripamonti M, Pezzoni G, Pesenti E, Pastori A, Farao M, Bargiotti A, Suarato A, Spreafico F, Grandi M (1992) In vivo anti-tumour activity of FCE 23762, a methoxy-morpholinyl derivative of doxorubicin active on doxorubicin-resistant tumour cells. Br J Cancer 65: 703
14. Wassermann K, Newman RA, Davis FM, Mullins TD, Rose KM (1988) Selective inhibition of human ribosomal gene transcription by the morpholinyl anthracyclines cyanomorpholinyl- and morpholinyldoxorubicin. Cancer Res 48: 4101
15. Wassermann K, Markovits J, Jaxel C, Capranico G, Kohn KW, Pommier Y (1990) Effects of morpholinyl doxorubicins, doxorubicin, and actinomycin D on mammalian DNA topoisomerase I and II. Mol Pharmacol 38: 38
16. Scudder SA, Brown JM, Sikic BI (1988) DNA cross-linking and cytotoxicity of the alkylating cyanomorpholino derivative of doxorubicin in multidrug-resistant cells. J Natl Cancer Inst 16: 1294
17. Lau DHM, Durán GE, Sikic BI (1992) Characterization of covalent DNA binding of morpholino and cyanomorpholino derivatives of doxorubicin. J Natl Cancer Inst 84: 1587
18. Chuang LF, Chuang RY, Acton EM, Israel M, Yu M (1987) Effect of morpholinyl-adriamycin analogs and adriamycin on the activities of DNA polymerase α and RNA polymerase II in chicken leukemia cells. J Pharmacol Exp Ther 242: 372
19. Zijlstra JG, Vries EGE de, Mulder NH (1987) Multifactorial drug resistance in an adriamycin-resistant human small cell lung carcinoma cell line. Cancer Res 47: 1780
20. De Jong S, Zijlstra JG, Vries EGE de, Mulder NH (1990) Reduced DNA topoisomerase II activity and drug-induced DNA cleavage activity in extracts from a human small cell lung carcinoma cell line. Cancer Res 50: 304
21. Scheper RJ, Broxterman HJ, Scheffer GL, Kaaijk P, Dalton WS, Heijningen THM van, Kalken C van, Slovak ML, Vries EGE de, Valk P van der, Meijer CJLM, Pinedo HM (1993) Overexpression of M_r 110,000 vesicular protein in non-P-glycoprotein-mediated multidrug resistance. Cancer Res 53: 1475
22. Versantvoort CHM, Broxterman HJ, Pinedo HM, Vries EGE de, Feller N, Kuiper CM, Lankelma J (1992) Energy-dependent processes involved in reduced drug accumulation in multidrug-resistant human lung cancer cell lines without P-glycoprotein expression. Cancer Res 52: 17
23. Zaman GJR, Versantvoort CHM, Smit JJM, Eijdems EWHM, Haas M de, Smith AJ, Broxterman HJ, Mulder NH, Vries EGE de, Baas F, Borst P (1993) Analysis of the expression of MRP, the gene for a new putative transmembrane drug transporter, in human multidrug resistant lung cancer cell lines. Cancer Res 53: 1747
24. Hospers GAP, Mulder NH, Jong B de, Ley L de, Uges DRA, Fichtinger-Schepman AMJ, Scheper RJ, Vries EGE de (1988) Characterization of a human small cell lung carcinoma cell line with acquired cisplatin resistance in vitro. Cancer Res 48: 403
25. Meijer C, Mulder NH, Hospers GAP, Uges DRA, Vries EGE de (1990) The role of glutathione in resistance to cisplatin in a human small cell lung cancer cell line. Br J Cancer 62: 72
26. De Jong S, Zijlstra JG, Mulder NH, Vries EGE de (1991) Lack of cross-resistance to fostriecin in a human small cell lung carcinoma cell line showing topoisomerase II related drug resistance. Cancer Chemother Pharmacol 28: 461
27. Meijer C, Mulder NH, Timmer-Bosscha H, Peters WHM, De Vries EGE (1991) Combined in-vitro modulation of adriamycin resistance. Int J Cancer 49: 582
28. Van der Graaf WTA, Vries EGE de, Uges DRA, Nanninga AG, Meijer C, Vellenga E, Mulder POM, Mulder NH (1991) In vitro and in vivo modulation of multidrug resistance with amiodarone. Int J Cancer 48: 616
29. Cole SPC, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kurz EU, Duncan AMV, Deeley RG (1992) Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. Science 258: 1650
30. Cole SPC (1990) Patterns of cross-resistance in a multidrug-resistant small-cell lung carcinoma cell line. Cancer Chemother Pharmacol 26: 250
31. Lau DHM, Lewis AD, Ehsan MN, Sikic BI (1991) Multifactorial mechanisms associated with broad cross-resistance of ovarian carcinoma cells selected by cyanomorpholino doxorubicin. Cancer Res 51: 5181
32. Lau DHM, Ross KL, Sikic BI (1990) Paradoxal increase in DNA cross-linking in a human ovarian carcinoma cell line resistant to cyanomorpholino doxorubicin. Cancer Res 50: 4056
33. Lewis AD, Durán GE, Lau DH, Sikic BI (1992) Sensitization of drug resistant human ovarian cancer cells to cyanomorpholino doxorubicin (MRA-CN) by modulation of glutathione metabolism. Int J Radiat Oncol Biol Phys 22: 821
34. Majima H (1990) Phase I clinical study of MX2 (KRN-8602). Gan To Kagaku Ryoho 17: 359
35. Ogawa M, Tabata M, Horikoshi N, Inoue K, Mukaiyama T, Fukutani H, Hirano A, Muzunuma N, Itami S (1989) Phase I trial of 3′-deamino-3′-morpholino-13-deoxy-10-hydroxycarminomycin hydrochloride (KRN-8602). Proc Am Soc Clin Oncol 8: A238