Antimetastatic Effect of a Novel Indolocarbazole (NB-506) on IMC-HM Murine Tumor Cells Metastasized to the Liver

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IMC-HM cells were isolated from spontaneously induced ascitic IMC carcinoma cells that had been maintained intraperitoneally in CDF_1 mice. Metastasis to the liver of subcutaneously implanted IMC-HM cells was detected 10 days after implantation into the flanks of mice (day 10), but metastasis to other organs was limited. Thereafter, however, tumor cells spread rapidly to lymph nodes, lung, spleen, ovary and other organs, and the mice died on day 13 to 18. We report here, together with the properties of IMC-HM cells, the effects of adriamycin, cisplatin, etoposide and a new indolocarbazole antitumor compound (NB-506) on this model of metastasis. Although these anticancer agents all inhibited the growth of the subcutaneous tumors, their effects on the life span of the tumor-bearing mice varied. Treatment with NB-506, started on day 1, more than doubled the survival period at doses 30 mg/m^2 to 900 mg/m^2. Further, treatment with NB-506, started on day 4 after resection of the primary tumor, inhibited growth of the metastasized tumor in the liver and other organs. Etoposide also increased the life span at a limited range of doses. However, the life-prolonging effects of adriamycin and cisplatin were marginal. These results demonstrate that IMC-HM carcinoma is a good model for spontaneous metastasis to the liver followed by lethal spread to many organs. Moreover, NB-506 was found to be highly effective against the growth not only of subcutaneous tumors, but also of tumors metastasized to the liver.

Key words: Metastasis — IMC-HM — NB-506 — Liver tumor — Indolocarbazole

The efficacy of available antitumor drugs is not sufficient for treatment of most solid tumors and the usefulness of these drugs is in any case limited because of their narrow therapeutic index. It is necessary to discover and develop new antitumor compounds with greater efficacy against solid-organ tumors and malignant metastases at terminal stages, and with wider safety margins. There are some good models of metastasis such as the orthotopic implantation models developed by Fidler and his colleagues.[1-3] However, these models are not suitable for large-scale screening assays because of a requirement for surgery. For large-scale evaluation of compounds for antimetastatic efficacy, it is necessary to establish better experimental models for solid-organ tumors that metastasize spontaneously and rapidly.

We describe here the establishment of a malignant metastatic liver tumor model and the effects of a new therapeutic compound, NB-506. In the course of routine i.p. passage of murine spontaneously induced ascitic IMC carcinoma cells,[4] we happened on a subline that killed CDF_1 mice in 2 weeks after s.c. implantation into the flank. Very marked metastasis to the liver was detected, as well as to many other organs, even though mice implanted s.c. with the original IMC carcinoma cells usually

survived for more than 50 days. This spontaneously generated IMC-HM cell line was maintained in the peritoneal cavity of mice and transferred to culture in vitro. IMC-HM cells have been maintained for more than a year, and their metastatic potential is quite stable. This cell line can be used for studies of spontaneous metastasis to the liver that is followed by rapid dissemination throughout the body, as well as for studies of the efficacy of antitumor compounds. Using IMC-HM cells, we investigated the effects of various antitumor agents, including the new antitumor indolocarbazole compound NB-506,[5, 6] in vitro and in vivo.

MATERIALS AND METHODS

Mice Female CDF_1 (BALB/c × DBA/2) mice were purchased from Charles River Japan (Kanagawa). All mice were 5 to 6 weeks old at the start of each experiment.

Tumor Murine spontaneously induced ascitic IMC carcinoma cells,[5] for which the primary site is unknown, were kindly provided by Dr. M. Ishizuka of the Institute of Microbial Chemistry (Shizuoka). IMC-HM cells were isolated from IMC cells that had been maintained i.p. in CDF_1 mice. The isolated IMC-HM cells were not cloned. They were maintained in vitro in RPMI 1640 supplemented with 10% fetal calf serum and 20 μM 2-mercap-

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toethanol, as well as by passage through the peritoneal cavity of CDF₁ mice.

NB-506 and other chemicals NB-506, 6-N-formylamino-12,13-dihydro-1,11-dihydroxy-13-(β-D-glucopyranosyl)-5H-indolo[2,3-a]pyrrolo[3,4-c]carbazole-5,7(6H)-dione (Fig. 1), was synthesized at our institute from BE-13793C[7] by enzymatic insertion of a glucopyranosyl group and the chemical addition of a formylamino group.[8] Details of its synthesis will be published elsewhere. Adriamycin (ADM) was purchased from Kyowa Hakko Kogyo Co., Ltd. (Tokyo). Cisplatin (CDDP) and etoposide (VP-16) were purchased from Nippon Kayaku Co., Ltd. (Tokyo).

Histology Various organs and tissues from CDF₁ mice that had been implanted s.c. with 5×10^4 IMC-HM carcinoma cells were removed 10 or 13 days after implantation, fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin (HE).

Drug sensitivity test *in vitro* IMC-HM and IMC carcinoma cells were suspended (2×10^4 cells/ml) in RPMI 1640 supplemented with 10% fetal calf serum and 20 μM 2-mercaptoethanol and 0.05 ml of the suspension was dispensed into wells of a 96-well plate. After incubation at 37°C overnight, 0.05 ml of medium containing the serially diluted test compound was added to each well and the incubation was continued for another 72 h. Cell growth was measured by the colorimetric tetrazolium-formazan (MTT) assay.[9]
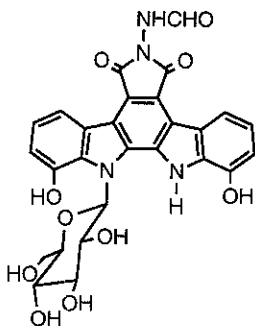

Fig. 1. The structure of NB-506 (6-N-formylamino-12,13-dihydro-1,11-dihydroxy-13-(β-D-glucopyranosyl)-5H-indolo-[2,3-a]pyrrolo[3,4-c]carbazole-5,7(6H)-dione).

Antitumor test *in vivo* IMC-HM cells (5×10^4 cells/mouse) were implanted into the flanks of CDF₁ female mice (control n=10 or 12; test, n=5 or 6). Test compounds were injected i.v. once a day for 10 consecutive days. Antimetastatic effects were evaluated by monitoring survival and tumor growth. Tumor growth was monitored periodically by recording tumor volume (V), calculated according to the formula[10] $V=(L \times W^2)/2$, where L=length and W=width. Synergy of activity was judged on the basis of survival period and number of completely cured animals.

Statistical analysis The significance of differences among experimental groups was examined by use of Mann-Whitney's U-test.

RESULTS

Biological properties of IMC-HM cells *in vitro* The sensitivity of IMC-HM cells to NB-506, ADM, CDDP and VP-16 was tested and compared with that of the parental IMC cells (Table I). Although the doubling time of both cell lines was almost the same, namely, 21.0 h for IMC cells and 21.2 h for IMC-HM cells, the sensitivity of IMC-HM cells to these drugs was 3 to 35 times higher than that of IMC cells.

Organ-specific metastasis Histological examinations were carried out of various organs and tissues, and the results are shown in Table II. Small or moderately sized focal metastases were found in livers of 4 of 5 mice killed 10 days after tumor implantation (Fig. 2A). Metastases to other sites were not detected with the exception of very slight focal infiltration of the renal cortex in 1 of 5 mice. One mouse, found dead on day 13, had marked metastasis to the liver (Fig. 2B) and multiple metastases to lymph nodes, lungs, spleen, ovaries, kidneys, and adrenal glands, as well as to other organs. These results suggested that IMC-HM cells implanted s.c. metastasize first to the liver and then spread very rapidly to other organs.

Timing of metastasis IMC-HM cells implanted s.c. into flanks of mice were surgically resected 3 or 7 days after implantation to investigate the timing of metastasis from the primary site of implantation. As shown in Table III, the survival of mice from which the primary tumors were removed on day 3 was almost the same as that of sham-

Table I. Cytotoxic Effects of NB-506, ADM, CDDP and VP-16 on IMC-HM and IMC Carcinoma Cells

Cells	Doubling time (h)	IC₅₀ (μg/ml)			
		NB-506	ADM	CDDP	VP-16
IMC-HM	21.2	0.011	0.021	0.023	0.0062
IMC	21.0	0.21	0.36	0.076	0.22

Table II. Sites of Metastasis of IMC-HM Cells Implanted s.c. in CDF$_1$ Female Mice

Organ	Metastasis in individual mice					
	Day 10					Day 13
Liver	+	+	++	±	−	+++
Lung	−	−	−	−	−	++
Kidney	−	±	−	−	−	+
Spleen	−	−	−	−	−	++
Adrenal	−	−	−	−	−	+
Lymph node	−	−	−	−	−	+++
Ovary	−	−	−	−	−	++
Pituitary gland	−	−	−	−	−	±
Gallbladder	−	−	−	−	−	±
Trachea	−	−	−	−	−	±

Key: −, not apparent; ±, very slight; +, slight; ++, moderate; +++, marked.

operated mice, indicating that metastasis had occurred within 3 days after tumor implantation.

Stability of metastatic potential The stability of the metastatic activity of IMC-HM cells was analyzed by determining the survival periods of mice that had been injected s.c. with 5×10^4 IMC-HM cells periodically over the course of one and half years. During this period, 8 examinations were conducted. The range of mean survival days in these 8 experiments was 14.7 to 17.1, with a mean $\pm$SD of 15.9 ± 1.7 days. Thus, the metastatic potential of IMC-HM cells remained stable for a long period of time.

Evaluation of anticancer agents *in vivo* CDF$_1$ mice were implanted s.c. with IMC-HM cells and treated i.v. with NB-506, ADM, CDDP and VP-16 from 1 day after tumor implantation. As shown in Table IV, NB-506 inhibited tumor growth at 9 mg/m^2 to 900 mg/m^2. The survival time of mice treated with NB-506 was significantly prolonged as compared to controls. NB-506 was effective over a wide range of doses, from 30 mg/m^2 to 900 mg/m^2. At 600 mg/m^2, all mice remained alive for 90 days. However, at 900 mg/m^2, 2 out of 5 mice died as a result of toxicity. The effects of ADM, CDDP and VP-16 were significantly less than those of NB-506, and no mouse was cured with these drugs.

The effects of treatment with NB-506, ADM, CDDP and VP-16 started 4 days after tumor implantation were examined to determine whether the prolonged survival was due to direct inhibition of growth of the primary tumors and/or to the suppression of metastases. Each treatment was carried out in combination with surgical resection of the primary tumors (Table V). The increase in the survival period of mice treated with NB-506, ADM, CDDP and VP-16 from day 4 was the same as that of mice treated from day 1, as shown in Table IV. Synergistic effects of NB-506 and surgery were observed
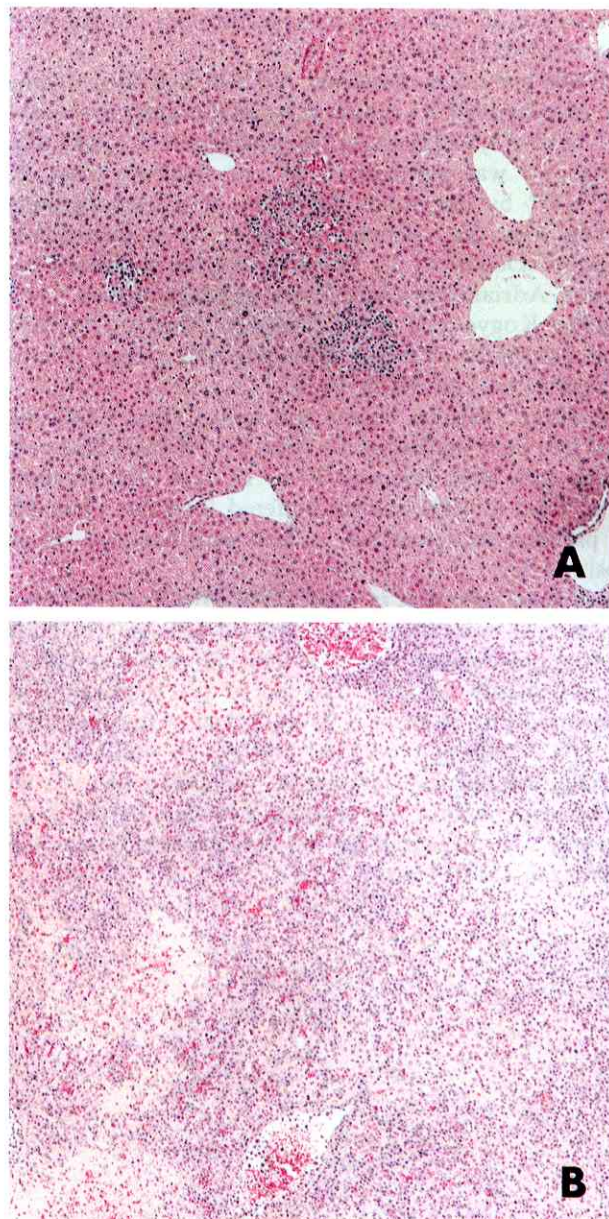

Fig. 2. Formation of colonies of IMC-HM cells in the liver (HE, ×60). Tumor-bearing mice were killed 10 days (A) and 13 days (B) after implantation of tumor cells.

at doses of 30 to 300 mg/m^2. At 90 mg/m^2 NB-506, 67% of mice were completely cured. ADM did not have a synergistic effect even at a subtoxic dose. CDDP and VP-16 had synergistic effects at 9 mg/m^2 only, but the effective dose was subtoxic and the number of the mice that were completely cured was lower than that with NB-506. The lack of effective synergistic action in the combination of resection with ADM, CDDP or VP-16 ap-

Table III. Effects of Resection of the Primary Tumor on the Survival Period in Mice Implanted s.c. with IMC-HM Cells

Surgical treatment	Treatment day[a]	Mean survival (days±SD)
Exp. 1		
None	—	15.3±1.0
Sham operation	7	14.2±1.0
Resection	3	15.6±3.0
Resection	7	14.5±1.5
Exp. 2		
None	—	15.6±1.3
Sham operation	3	14.8±0.7
Resection	3	17.5±4.5

CDF_1 female mice were implanted s.c. with 5×10^4 IMC-HM cells.
a) Tumors were implanted on day 0.

Table IV. Effects of NB-506, ADM, CDDP and VP-16 on the Growth of Primary Tumors and the Survival Period of Mice Implanted s.c. with IMC-HM Cells

Drug	Dose (mg/m²)	Primary tumor volume (cm³±SD)	Survival time (days±SD)	Survivors on day 90
Control	0×10	1.25±0.23	14.7±1.4	0/10
NB-506	9×10	0.32±0.11[a]	18.6±1.1[a]	0/5
	30×10	0.03±0.00[a]	>36.8±26.4[a]	1/5
	90×10	0.02±0.02[a]	39.0±1.7[a]	0/5
	300×10	0.00±0.00[a]	>54.2±5.5[a]	1/5
	600×10	0.00±0.00[a]	>60.0±0.0[a]	5/5
	900×10	0.00±0.00[a]	>37.0±47.9	3/5
ADM	0.9×10	1.26±0.17	14.8±0.4	0/5
	3×10	0.97±0.31	17.0±1.9	0/5
	9×10	0.22±0.04[a]	24.0±2.0[a]	0/5
	30×10	—	10.8±0.4[a]	0/5
CDDP	0.3×10	1.21±0.26	16.8±0.4[b]	0/5
	0.9×10	0.52±0.06[a]	19.2±0.8[a]	0/5
	3×10	0.06±0.03[a]	25.0±0.7[a]	0/5
	9×10	0.01±0.01[a]	39.8±6.4[a]	0/5
	30×10	—	6.4±0.5[a]	0/5
VP-16	0.9×10	0.98±0.35	16.0±1.2	0/5
	3×10	0.46±0.16[a]	19.2±1.1[a]	0/5
	9×10	0.04±0.04[a]	25.0±1.4[a]	0/5
	30×10	0.03±0.03[a]	56.2±10.0[a]	0/5
	90×10	—	11.0±1.2[a]	0/5

a) Significantly different from the control ($P<0.01$).
b) Significantly different from the control ($P<0.05$).
—, All mice died as a result of toxicity. CDF_1 mice implanted s.c. with IMC-HM cells on day 0 were treated with drugs i.v. once a day for 10 consecutive days from day 1. The size of primary tumors was determined on day 13.

Table V. Life-span-increasing Effects of NB-506 with and without Resection of Primary Solid IMC-HM Tumors

Drug	Dose (mg/m²)	T/C% of mean survival days (S/A[a])			
		Resection +		Resection −	
Control		100	(0/12)	100	(0/12)
NB-506	9	143[b]	(0/6)	129[b]	(0/5)
	30	>230[b]	(2/6)	201[b]	(0/6)
	90	>322[b]	(4/6)	256[b]	(0/6)
	300	>299[b]	(1/5)	256[b]	(0/6)
ADM	0.9	103	(0/6)	111[c]	(0/5)
	3	114[b]	(0/6)	114[b]	(0/5)
	9	142[b]	(0/6)	138[b]	(0/5)
CDDP	0.9	128[b]	(0/6)	128[b]	(0/5)
	3	154[b]	(0/6)	148[b]	(0/5)
	9	>253[b]	(1/6)	191[b]	(0/5)
VP-16	0.9	119[b]	(0/6)	109[c]	(0/5)
	3	130[b]	(0/6)	124[b]	(0/5)
	9	>180[b]	(1/6)	158[b]	(0/5)
	30	197[b]	(0/6)	184[b]	(0/5)

a) Number of surviving mice on day 60/number of mice tested.
b) Significantly different from the control ($P<0.01$).
c) Significantly different from the control ($P<0.05$).
Surgery was performed on day 3 and the drug was administered i.v. once a day for 10 consecutive days from day 4. Duration of survival of mice that were alive on day 60 was taken as 60 days. Mean survival days for operated controls, sham-operated controls and non-operated controls were 16.1, 15.8 and 15.8, respectively.

cells, and this antimetastatic effect of NB-506 was much greater than those of the other anticancer agents tested.

DISCUSSION

Although it is obvious that testing the efficacy of anticancer agents in animal models with tumors in specific organs is preferable to testing in animal models with subcutaneously transplanted tumors, growth-inhibitory effects on s.c. tumor nodules are conventionally used to assess antitumor drugs.[11–13] These s.c. tumor models are easy to generate. Organ-specific tumor models have been constructed by surgically implanting tumor cells in a target organ[1, 14, 15] or by injecting them into the spleen or a lung.[2, 3, 16, 17] However, these models are not suitable for large-scale screening assays. Therefore, there is a need for organ-tumor models that do not require surgery and are reproducible. Moreover, it is known that the antitumor effects of some compounds depend on the sites at which tumor cells grow.[18] In addition, models of metastasis are necessary for evaluation of antimetastatic drugs.

IMC-HM cells are particularly useful because they produce liver tumors by spontaneous metastasis from subcutaneous implants and can be used to evaluate the effects of test compounds on both the process of metasta-

peared to be related to the low potency of growth inhibition in the organs. Our results indicate that NB-506 markedly inhibited the proliferation of metastasized tumor

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sis and the growth of tumors that have metastasized to the liver. Other well-studied and widely adapted metastatic murine tumor cell lines, such as B16/BL6 and B16/F10 melanoma cells[19] and Lewis lung carcinoma cells, metastasize to the lung, but not the liver. It should be noted that, in B16 melanoma models, the tumors are injected i.v. and, thus, they do not reflect the most prevalent metastatic processes in real human cancers. To date, experimental models of metastasis to the liver have been limited in number.[20] Thus, the metastatic potential of IMC-HM should be very useful for evaluation of antimetastatic drugs in large-scale screening assays.

CDF$_1$ mice implanted s.c. with 5×10^4 IMC-HM cells were asymptomatic until 10 days after implantation of the cells, when no metastases were yet detectable by gross examination. Thereafter, tumor cells proliferated very rapidly in the liver and killed all mice as a result of metastasis to multiple organs by day 13. Since the metastases found histologically on day 10 were metastases in the liver, it seems that the first site of metastasis was the liver and that the later malignant tumors resulted from the spread of tumor cells deposited in the liver. This speculation is supported by the fact that metastasis to organs other than the liver occurred to almost the same extent even when primary tumors implanted s.c. were resected 3 days after implantation of the cells. However, it is also possible that growth of metastasized tumors in organs other than the liver was slower than that in the liver and, therefore, these tumors became apparent later, even though the original metastasis occurred at the same time as that to the liver. The rapid expansion of metastasis, beginning after 10 days, was remarkable and similar to malignant metastasis observed at terminal stages in man.

It should be noted that the metastatic potential of IMC-HM was found to be very stable. The interexperimental and intraexperimental fluctuations in survival times of mice implanted s.c. with IMC-HM cells were narrow, and reproducible results were obtained over the course of 18 months.

The usefulness of IMC-HM cells as a model for the evaluation of antitumor compounds was examined by comparing the efficacy of commonly used compounds, namely, ADM, CDDP, VP-16, and NB-506. When mice were treated from 1 day after tumor implantation, NB-506 was most effective in inhibiting the growth of the primary tumor and it increased the survival times over a wide range of doses. The effects of ADM, CDDP and VP-16 on the survival period of mice were significantly less. Metastasis involves the release of cells from the primary tumor, dissemination to distant organs, infiltration into the stroma of those organs, and manifestation of micro-metastasized tumors at these sites. Our IMC-HM model includes all of these steps. Therefore, it was not clear initially whether the increased survival caused by

the anticancer agents tested was due to inhibition of the growth of primary tumors or to inhibition of some step(s) in the metastatic process. To resolve this question, treatment with NB-506 was started 4 days after tumor implantation, in combination with resection of the primary tumor. If the increased survival due to NB-506 from 1 day after tumor implantation (Table II) had been solely due to inhibition of primary tumors, then treatment starting 4 days after tumor implantation would be expected to be ineffective. However, NB-506 was found to be effective even when administered from day 4. In combination with removal of the original tumor, NB-506 gave even better results. Thus, NB-506 not only inhibited growth of the original tumor, but also inhibited the manifestation of micro-metastasized tumors in the liver and other organs. In addition, the growth-inhibiting activity of NB-506 against the micro-metastasized tumors was more potent than that against the primary tumor.

At present, it is unknown whether metastasis of IMC-HM tumor cells to the liver and other organs is due to circulation of tumor cells through the blood-vascular system or the lymphatic system. Nevertheless, this metastasis model seems better than models in which tumor cells are directly injected i.v. since the tumor cells are implanted into the flanks of mice. In our model of metastasis, NB-506 clearly inhibited the manifestation of micro-metastasized tumors in the liver and other organs, while other well-known anticancer agents were not as effective, even though they significantly inhibited the growth of the primary implanted tumors.

The release of cells from a primary tumor and the attachment of released tumor cells to other organs are early events in metastasis. Many investigations have been undertaken in attempts to inhibit the adhesion process of released tumor cells to other organs and, thus, to inhibit metastasis. However, in the case of real human cancers, these early events of metastasis have often already occurred prior to surgery. In many cases, metastasis cannot be detected histologically, but micro-metastasized tumors already exist in distant organs. Therefore, to inhibit metastasis in actual human cancers it is important not so much to inhibit adhesion of tumor cells, but rather to inhibit the manifestation or growth of micro-metastasized tumors. Thus, the model of metastasis described herein should be very useful for the development of practical antimetastatic agents.

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