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ORIGINAL ARTICLE

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# Treatment of VX2 carcinoma implanted in the liver with arterial and intraperitoneal administration of oily anticancer agents

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**Abstract** *Purpose*: Long-term survival and cure cannot be achieved in patients with unresectable, advanced abdominal cancer, because no chemotherapeutic treatment has definite antitumor activity for malignant solid tumor and its dissemination. In this study, arterial and intraperitoneal administration of oily anticancer agents, which have properties that permit targeted chemotherapy for VX2 carcinoma implanted in the liver, was attempted to achieve long-term survival. *Materials and methods*: Rabbits bearing VX2 tumors in the liver measuring 1–2 cm in diameter received an arterial injection of 0.2 ml of nitrogen mustard *N*-Oxide ($HN_2$-O) dissolved in Lipiodol (7.5 mg/ml), a newly developed oily anticancer agent, for the tumor and an intraperitoneal injection of a cocktail of oily anticancer agents for the prevention of intraperitoneal dissemination. *Results*: Twelve out of thirteen rabbits survived and VX2 cancer was not observed in these 12 rabbits. The controls received a sham operation, an intraperitoneal injection of the cocktail of oily anticancer agents alone, or an arterial injection of $HN_2$-O/Lipiodol alone. In these control groups, 27 out of 29 rabbits died of cancer. To examine the dose form for arterial injection, 14 rabbits received an arterial injection of the simple mixture of $HN_2$-O dissolved in physiological saline and Lipiodol, with an additional intraperitoneal injection of the cocktail. Eight of these 14 rabbits died of enlargement of the hepatic tumor and peritoneal dissemination. *Conclusion*: Long-term survival and cure was achieved in almost all rabbits bearing VX2 tumor in the liver by simultaneous arterial and intraperitoneal injection of oily anticancer agents.

**Key words** Targeted chemotherapy · VX2 tumor · Lipiodol ultrafluid

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## Introduction

Many patients with advanced or recurrent cancer in the abdominal cavity die of metastatic liver cancer or panperitonitis carcinomatosa or both. The clinical appearance of this disease is similar to the appearance of the disease in rabbits bearing VX2 tumor in the liver. In earlier studies, even if VX2 tumor in the liver shrank or disappeared after arterial injection of oily anticancer agents such as mitomycin C/Lipiodol and doxorubicin/Lipiodol, many rabbits died of peritoneal dissemination. Therefore, long-term survival of rabbits bearing VX2 tumor in the liver has been unlikely. For successful treatment of this advanced cancer, solid tumor and peritoneal dissemination must be treated simultaneously.

We have reported that a lipid lymphographic contrast medium, Lipiodol ultrafluid (Lipiodol), when administered arterially, was retained selectively for more than 3 months in hepatocellular carcinoma and for more than 3 weeks in metastatic liver cancer and other solid malignant tumors [1, 4, 8, 9]. The preparation that enabled targeted cancer chemotherapy with Lipiodol as a carrier of anticancer agents had the characteristics that the anticancer agent was dissolved in and was stable in Lipiodol and that it diffused out from it very slowly. The amount of anticancer agent that diffused out from the Lipiodol while it remained only in the tumor was the amount of anticancer agent delivered selectively (targeted) to the tumor. These preparations were named oily anticancer agents [4]. We have reported clinical and experimental results showing that solid malignant

tumors can be successfully treated by targeted cancer chemotherapy with oily anticancer agents such as SMANCS/Lipiodol [4, 7, 8], mitomycin C (MMC)/Lipiodol, doxorubicin/Lipiodol, aclarubicin/Lipiodol, and vinblastine/Lipiodol [4, 6, 9, 10, 12]. This type of targeted chemotherapy has been reported to be available for various kinds of cancer [8]; clinically speaking, many kinds of oily cancer agents were thought to be necessary, but one that is less expensive and more effective is still needed. Intraperitoneal administration of oily anticancer agents also had an anticancer effect on peritoneal dissemination, because of the long-lasting release of the drug from the oily anticancer agents into the peritoneal cavity [2, 3].

In this study, we used simultaneous arterial and intraperitoneal administration of a cheaper and newly developed oily anticancer agent, $HN_2$-O/Lipiodol, for rabbits bearing VX2 tumor in the liver, in an attempt to produce long-term survival. To prevent peritoneal dissemination, a mixture of oily anticancer agents that had had clear anticancer effects on peritoneal dissemination in a preliminary study (T. Konno, unpublished work) was used.

## Materials and methods

### Drugs

Lipiodol is a sterile iodine addition product of the ethyl ester of the fatty acid obtained from poppy seed oil (iodine concentration 38 w/w%; Laboratoire Guerbet, Aulnay-Sous-Bois, France).

The oily anticancer agents used for arterial injection were:

a. Newly developed $HN_2$-O/Lipiodol, which is less expensive than others (1/100 of the cost of SMANCS/Lipiodol). It was prepared by first dissolving $HN_2$-O in chloroform and then dissolving this mixture in Lipiodol contrast medium that contained lecithin at 8 times the weight of $HN_2$-O. The solvent chloroform was then removed. The endpoint for removal of the solvent was determined by the volume of the product and the solvent removed. Residual chloroform as measured by column chromatography was less than 5% by volume. Each milliliter of $HN_2$-O/Lipiodol contained 7.5 mg of $HN_2$-O. The $HN_2$-O/Lipiodol was used within 1 week of preparation.
b. A cocktail of oily anticancer agents, because it had had clear anticancer effects on peritoneal dissemination in a preliminary study (T. Konno, unpublished work). MMC, aclarubicin, and carmustine (BCNU) were dissolved in Lipiodol (solution), and styrene maleic acid neocarzinostatin (SMANCS) was dispersed in Lipiodol (suspension). One milliliter of the Lipiodol cocktail contained 0.4 mg of SMANCS, 1.6 mg of MMC, 5 mg of aclarubicin, and 5.5 mg of BCNU [5].

### Experimental animals and anesthesia

We used 56 New Zealand White rabbits of either sex. The mean body weight was 1.6 kg. General anesthesia was induced by intravenous injection of pentobarbital sodium (30 mg/kg) during both the laparotomy for tumor inoculation and the arterial and intraperitoneal injections of the drugs. Kumamoto University, Japan, guidelines for animal care were followed.

### Tumor inoculation

Transplantable anaplastic VX2 carcinoma that had originated from spontaneously transformed Shope papilloma, which was first described by Rous and Beard [13], was used. The tumor was solid up to a diameter of 1.3 cm in the liver. Larger tumors had necrotic foci that tended to become confluent so that a large area of central necrosis formed. The VX2 tumor cell line was maintained by successive transplantation into the liver of rabbits. A 1.5-mm section of the VX2 tumor was inserted into the subcapsular parenchyma of the left anterior lobe of the liver. Two weeks later, the animals underwent laparotomy and rabbits with tumors measuring 1–2 cm in diameter were used for the experiments.

### Drug administration

#### *Arterial injection of oily anticancer agents*

The hepatoduodenal ligament was stretched by pulling the stomach to the caudal side so that the hepatic artery could be seen. All the drugs tested were injected within about 15 s as a bolus into the nonoccluded proper hepatic artery by use of a special fine needle (outer diameter 0.21 mm, inner diameter 0.12 mm) connected to a 10-cm-long polyethylene tube. While the drugs were being injected, the gastroduodenal artery was clamped with bulldog forceps to prevent flow of the drug into the duodenum. After the injection, blood flow in the hepatic artery was confirmed as sustained.

#### *Intraperitoneal injection of the cocktail of oily anticancer agents*

For each animal, 0.5 ml of the cocktail was injected intraperitoneally immediately before closure of the operative abdominal wound.

### Test for antitumor activity

Five groups of rabbits were given different drug preparations. The rabbits in group 1 ($n = 12$) underwent a sham operation, with puncture of the proper hepatic artery but no drug administration. The rabbits in group 2 ($n = 9$) received only intraperitoneal injection of 0.5 ml of the cocktail of oily anticancer agents. The rabbits in group 3 ($n = 8$) received only arterial injection of 0.2 ml of $HN_2$-O/Lipiodol (7.5 mg/ml). Rabbits in group 4 ($n = 14$) received intraperitoneal injection of 0.5 ml of the cocktail and arterial injection of 0.4 ml of the simple mixture of 0.2 ml of Lipiodol and 0.2 ml of $HN_2$-O dissolved in physiological saline solution (7.5 mg/ml). The rabbits in group 5 ($n = 13$) received intraperitoneal injection of 0.5 ml of the cocktail and arterial injection of 0.2 ml of $HN_2$-O/Lipiodol (7.5 mg/ml).

A preliminary experiment revealed that VX2 tumors receiving intraarterial injection of 0.2 ml of $HN_2$-O/Lipiodol (10 mg/ml) were completely necrotic, accompanied by spotty necrosis of normal parenchyma of the liver; and the complete necrosis of VX2 tumors receiving intraarterial injection of 0.2 ml of $HN_2$-O/Lipiodol (5 mg/ml) was observed in 70% of rabbits. Therefore, we used 0.2 ml of $HN_2$-O/Lipiodol (7.5 mg/ml) for arterial injection therapy in this experiment.

All the animals were allowed to survive for at least 300 days. During the entire experimental period, all the animals were examined daily for any abnormality and mortality. All dead rabbits were examined by autopsy. Animals that survived for more than 300 days after the injection of the drugs were killed. All livers were removed and fixed in 20% formalin.

The antitumor effects on VX2 carcinoma were evaluated by assessment of the survival period, histological findings of the tumor in the liver, and peritoneal dissemination. A preliminary study revealed that peritoneal dissemination was observed in the early stage as small, white nodules of 1–2 mm in the greater omentum; various tumors of 1–150 mm were observed throughout the abdominal cavity in the advanced stage.

Statistical analysis

Survival curves were determined by the Kaplan-Meier method. The survival period data were expressed as mean ± SE. The numbers of tumor-free rabbits were analyzed by mean of Fisher's exact probability test. A *P*-value of less than 0.05 was regarded as representing a significant difference.

## Results

### Antitumor activity against hepatic tumor and prevention of peritoneal dissemination

In group 1 (sham operation), all hepatic tumors were enlarged at autopsy and peritoneal dissemination was observed in 11 out of 12 rabbits. In group 2 (intraperitoneal injection of oily anticancer agents alone), enlargement of hepatic tumors was observed in all nine rabbits, and peritoneal dissemination was observed in eight of these nine rabbits. In group 3 (arterial injection of $HN_2$-O/Lipiodol), enlarged hepatic tumors and peritoneal dissemination were observed in two out of eight rabbits. In the remaining six rabbits, complete necrosis of the hepatic tumor was observed at autopsy. Peritoneal dissemination was found in four of these six rabbits (Fig. 1). In group 4 (arterial injection of the simple mixture plus intraperitoneal injection of oily anticancer agents), enlargement of hepatic tumors and peritoneal dissemination were observed in 8 of 14 rabbits. In the remaining 6 rabbits, the tumor had disappeared at death and peritoneal dissemination was not observed. In group 5 (arterial injection of $HN_2$-O/Lipiodol plus intraperitoneal injection of the oily anticancer agents), hepatic tumor enlargement and peritoneal dissemination were observed in 1 out of 13 rabbits. The hepatic tumor had disappeared and no peritoneal dissemination was found in the remaining 12 rabbits at autopsy. The number of tumor-free rabbits was significantly better than that of group 4 (Table 1). Peritoneal disseminations observed in this series were at advanced stage of peritoneal dissemination (Fig. 1).

### Survival

All the rabbits in group 1 died of cancer within 100 days of the sham operation and mean survival time was $61 \pm 7$ days. Nine rabbits in group 2 died of cancer within 180 days of the intraperitoneal injection and

**Fig. 1A–D** Peritoneal dissemination. **A** Peritoneal dissemination at low magnification. Original magnification ×5. **B** At higher magnification, the viable tumor cells are arranged in sheet-like or solid nests. Original magnification ×100. **C** Appearance of VX2 tumor 25 days after treatment in group 3 (*T* complete necrosis of hepatic tumor, *P* peritoneal dissemination, *L* liver). *Bar* 10 mm. **D** Microscopic findings of complete necrosis of hepatic tumor in **C**. Original magnification ×100

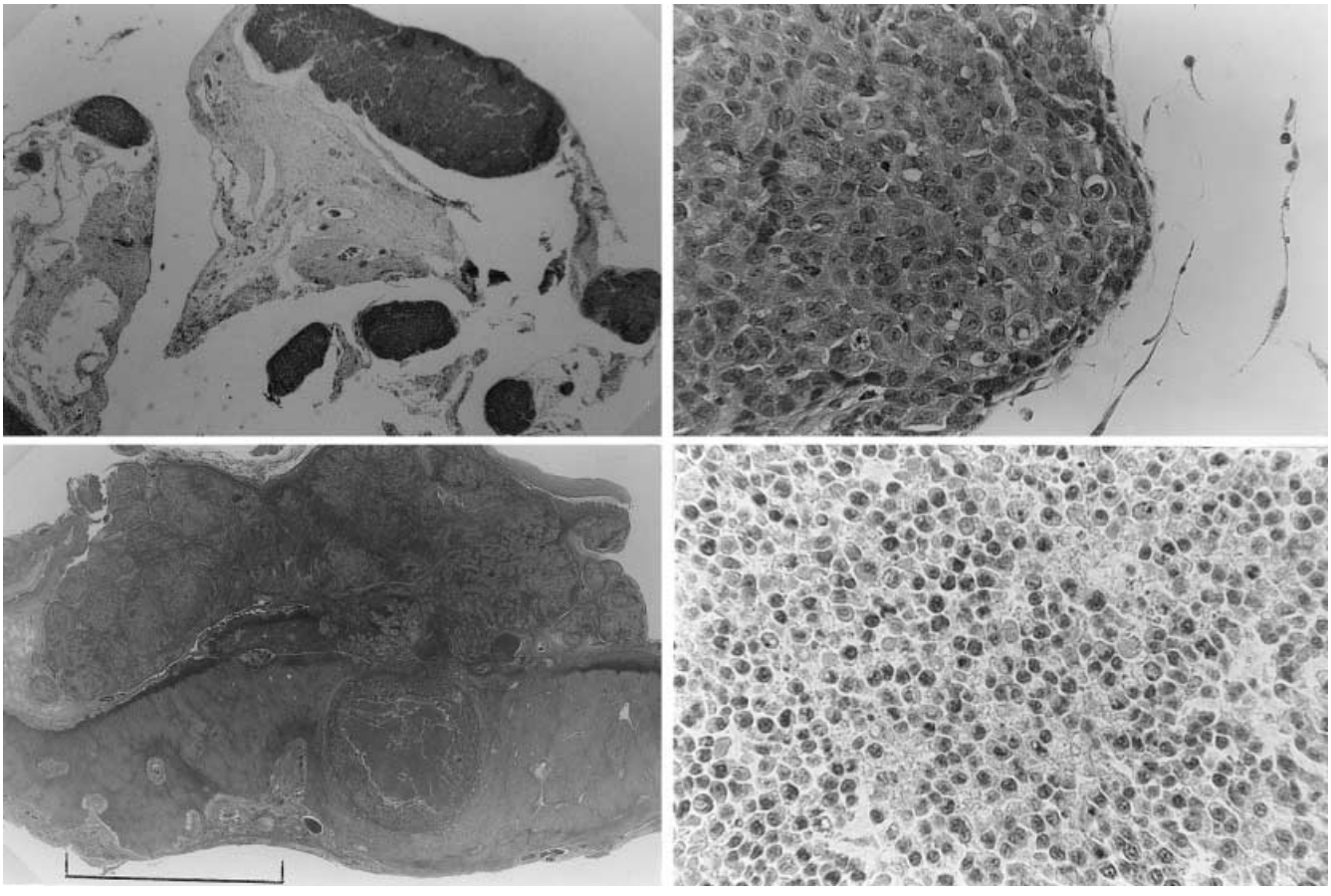

**Table 1** Findings for hepatic tumor and peritoneal dissemination at autopsy

| Group | Animals (*n*) | Findings in animals at autopsy or death (*n*) | | | | Animals surviving 300 days and tumor-free* | |
|---|---|---|---|---|---|---|---|
| | | Liver | | Abdominal cavity | | *n* | % |
| | | Enlarged tumor | Tumor-free | Carcinomatosis | Tumor-free | | |
| 1. Sham operation | 12 | 12 | 0 | 11 | 1 | 0 | 0 |
| 2. Intraperitoneal injection alone | 9 | 9 | 0 | 8 | 0 | 0 | 0 |
| 3. Arterial injection of $HN_2$-O/Lipiodol | 8 | 2 | 6 | 6 | 2 | 2 | 25 |
| 4. Arterial injection of $HN_2$-O/saline with Lipiodol plus intraperitoneal injection | 14 | 8 | 6 | 8 | 6 | 6 | 42 |
| 5. Arterial injection of $HN_2$-O/Lipiodol plus intraperitoneal injection | 13 | 1 | 12 | 1 | 12 | 12 | 92 |

* Not significant for groups 1, 2, and 3. The survival rate of group 5 was significantly better than that of the other 4 groups: $P < 0.0005$

mean survival time was $88 \pm 14$ days, but there was no significant difference in survival time between groups 1 and 2. In group 3, six of eight rabbits died of peritoneal dissemination; the remaining two rabbits survived for more than 300 days, and autopsy revealed no cancerous lesions in these two rabbits. Mean survival time of group 3 was $123 \pm 41$ days. Eight rabbits of fourteen in group 4 died of cancer within 75 days; the remaining 6 rabbits survived for more than 300 days after treatment. Mean survival time of group 4 was $118 \pm 81$ days. Survival of group 4 was significantly better than that of group 1. Twelve out of thirteen rabbits in group 5 survived for more than 300 days; 1 died of cancer at 29 days after drug injection. Mean survival time of group 5 was $279 \pm 21$ days. Thus, survival in group 5 was significantly better than that of any other group, including group 4 (Fig. 2).

### Microscopic examination

VX2 tumor cells were observed in enlarged hepatic tumors, namely, in all rabbits in group 1 and group 2, in 2 out of 8 rabbits in group 3, in 8 of 14 rabbits in group 4, and in 1 out of 13 rabbits in group 5. VX2 tumor cells were also observed in all peritoneal disseminations.

In rabbits surviving for more than 300 days after the injection, VX2 tumor was not present in either the liver or the abdominal cavity. Microscopic examination revealed that the tumor was completely replaced by scar tissue, consisting of fibrosis and accumulated, large foamy macrophages (Fig. 3).

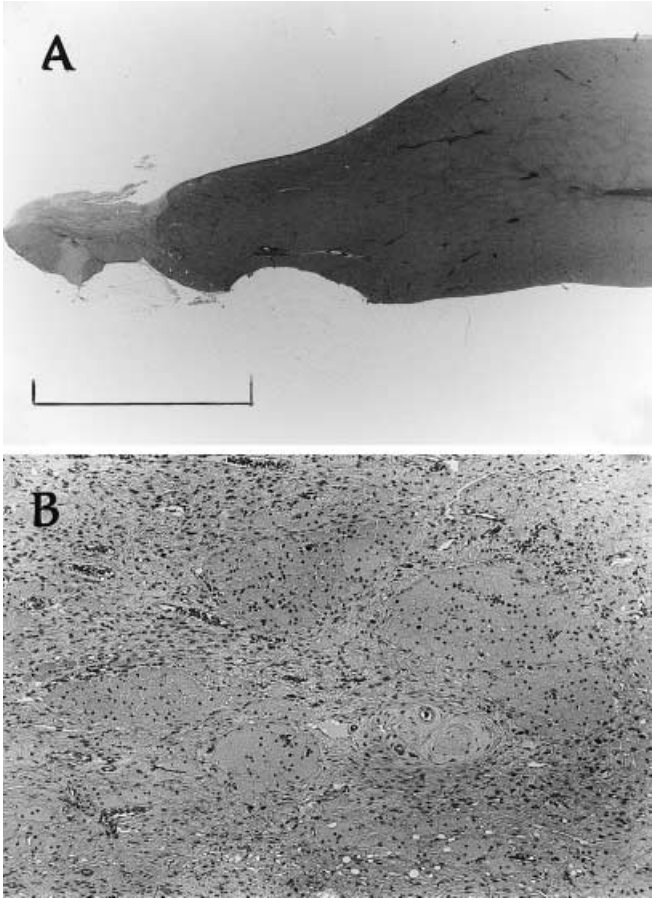


**Fig. 2A,B** Appearance of the liver lacking cancerous lesions 300 days after treatment in group 5. **A** Low magnification. The VX2-implanted region in the liver is replaced by scar tissue (*arrow*). *Bar* 10 mm. **B** Higher magnification of the scar tissue, characterized by fibrosis with the accumulation of large foamy macrophages. Original magnification ×25

## Discussion

Arterial injection of an inexpensive and newly developed oily anticancer agent, $HN_2$-O/Lipiodol, produced clear anticancer effects against VX2 tumor, with complete necrosis of hepatic tumors being achieved in 18 out of 21 rabbits. This result is similar to results with previously developed oily anticancer agents in our clinic [4, 10, 11, 12] (Table 2).

In our pharmacokinetic study of oily anticancer agents, we found a high drug concentration in the pleural or peritoneal cavity for a few weeks and a low drug concentration in the blood stream, and intracavitary administration of oily anticancer agents showed

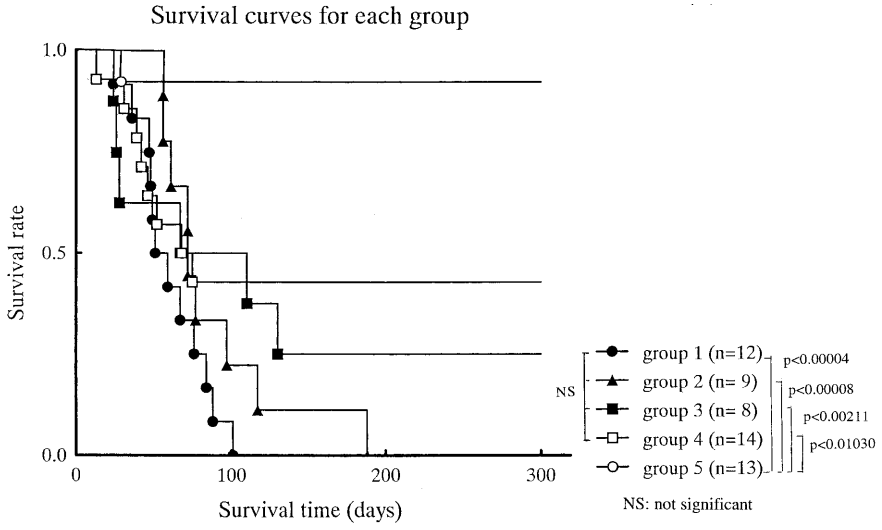


**Fig. 3** Survival curves for each group

definite antitumor activity against peritoneal dissemination [12].

However, intraperitoneal injection of oily anticancer agents alone in rabbits with enlarged tumor did not prevent peritoneal dissemination. Release of the anticancer agents from Lipiodol continued for a few weeks, but thereafter, continuous dissemination of cancer cells from the enlarged tumor was thought to occur.

In six of eight rabbits treated with arterial injection of $HN_2$-O/Lipiodol, the hepatic tumor disappeared. Four of these six rabbits died of peritoneal dissemination. These results suggest that the rabbits had undetectable peritoneal dissemination 2 weeks after the inoculation, at a tumor diameter of 10–20 mm.

Six of fourteen rabbits in group 4 survived 300 days after arterial injection of $HN_2$-O/saline plus Lipiodol and intraperitoneal injection of oily anticancer agents. The remaining 8 rabbits died of cancer. Twelve out of thirteen rabbits survived 300 days after arterial injection of the oily anticancer agents, $HN_2$-O/Lipiodol and intraperitoneal injection of oily anticancer agents; there was no evidence of VX2 cancer in the liver and peritoneal cavity at autopsy in group 5. Although the same drug and the same dosage were used in these two groups, their survival periods were significantly different. This difference may be explained by whether or not tumor targeting could be achieved.

**Table 2** Complete necrosis of VX2 carcinoma after arterial injection of oily anticancer agents. Single injection of 0.2 ml into rabbits bearing tumors of 10–20 mm in diameter

| Drugs | Organ | Number of animals with complete necrosis/total number animals | |
|---|---|---|---|
| | | *n* | % |
| Mitomycin C (4 mg/ml) [4] | Liver | 9/10 | 90 |
| Mitomycin C (3 mg/ml) [9] | Large bowel | 8/10 | 80 |
| Aclasinomycin (12.5 mg/ml) [4] | Liver | 6/8 | 75 |
| SMANCS (1 mg/ml) [3, 5] | Liver | 6/15 | 40 |
| Doxorubicin (2 mg/ml) [4] | Liver | 5/7 | 71 |
| Vinblastine (5 mg/ml) [6] | Liver | 11/11 | 100 |

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