3. Studies of Cancer in Animals

3.1 Infection with *Opisthorchis viverrini* alone

Hamster: In a histopathological study, a group of 30 male Syrian golden hamsters, three to four weeks of age, were infected with 100 metacercariae of *O. viverrini* by intragastric intubation. A group of 18 untreated hamsters served as controls. Five treated and three control animals were killed at 3, 7, 15, 30, 45 and 154 days after infection. The early pathological changes consisted of an acute inflammatory reaction involving the second-order bile ducts and portal connective tissue as well as focal coagulation necrosis of the liver lobules. As the liver flukes developed into adults (after 28 days), they induced hyperplasia, 'adenomatous formation' of the bile-duct epithelium, ductular proliferation and multilobular cirrhosis (Bhamarapravati *et al.*, 1978). [The Working Group noted the short duration of the study in relation to the lifespan of the animals, as it is possible that tumours could have developed in the animals if they had been allowed to live.]

As part of combination experiments (see section 3.2), a control group of 50 male Syrian golden hamsters, six to eight weeks of age, was given 50 *O. viverrini* metacercariae intragastrically and followed for 76 weeks. No bile-duct carcinoma was found (Flavell & Lucas, 1982, 1983).

Other groups of hamsters administered *O. viverrini* metacercariae alone as controls in combination experiments also had no bile-duct tumours after observation periods ranging from 22 to 45 weeks (Thamavit *et al.*, 1978, 1987a,b, 1988a,b, 1992a,b, 1994). In a further study (Thamavit *et al.*, 1993), a group of 18 female Syrian golden hamsters, six to eight weeks of age, received 60 *O. viverrini* metacercariae by intragastric intubation; 15 females received no treatment. Ten treated animals developed cholangiofibrosis and two developed cholangiocarcinomas within 38 weeks. No tumour was observed among controls. The difference in tumour rate was not significant.

A total of 150 male and 150 female Syrian hamsters, six to eight weeks of age, were divided into four groups and were infected monthly for 10 months with 0 (20 males and 20 females), 13 (40 males and 40 females), 25 (40 males and 40 females) or 50 (50 males and 50 females) *O. viverrini* metacercariae per intragastric intubation. Animals were then maintained on basal diet until they were killed at the end of week 52. Ten monthly intragastric applications of 0, 13, 25 or 50 metacercariae resulted in pronounced proliferative and inflammatory lesions involving the first- and second-order ducts, in response to the presence of adult worms. Cholangiofibrosis was seen, but no neoplastic lesion was evident after one year (Thamavit *et al.*, 1995). [The Working Group noted the short duration of the study.]

Table 5. Case–control studies of the association between *Chlonorchis sinensis* infection and cholangiocarcinoma and hepatocellular carcinoma

Location	Period of study	Type of cancer	Cases		Controls		Method of assessing *O. sinensis* infection	RR	95% CI	Reference
			Method of ascertainment	No.	Definition	No.				
Hong Kong	1964–66	CCA HCC	Autopsy	17 83	Autopsied subjects without CCA or HCC	1384	Gross examination at autopsy	3.1[a] 0.73[a]	0.13–8.4 0.45–1.2	Gibson (1971)
Republic of Korea, Seoul and Pusan	1961–72	CCA HCC	Autopsy and surgery of subjects with liver disease	54 423	Subjects coming to autopsy or surgery with liver disease in whom cancer was not found	1348	Examination of liver tissue or stool samples	6.5 1.2	3.7–12 0.80–1.7	Kim *et al.* (1974)
Republic of Korea, Pusan	1963–74	CCA HCC	Consecutive series of patients diagnosed mainly in two hospitals	36 170	Subjects admitted to these hospitals with diseases other than of the liver	559	Examination of stool samples	6.0 1.1	2.8–13 0.65–1.7	Chung & Lee (1976)

Relative risks and 95% confidence intervals calculated by the Working Group. CCA, cholangiocarcinoma; HCC, hepatocellular carcinoma. The two last studies partially overlap.

[a]Adjusted for age and sex

3.2 Infection with *Opisthorchis viverrini* in combination with administration of known carcinogens

3.2.1 N-*Nitrosodimethylamine*

Hamster: Male Syrian golden hamsters, aged three to four weeks, were divided into four groups: 18 animals served as untreated controls; 21 animals received 0.0025% [25 mg/L] *N*-nitrosodimethylamine (NDMA) in the drinking-water starting from seven to eight weeks of age; 18 animals were infected with 100 *O. viverrini* metacercariae by intragastric intubation; and 21 animals were infected with *O. viverrini* and, four weeks later (as soon as the parasitic eggs were detected in faeces), received NDMA in the drinking-water. NDMA treatment was discontinued after 10 weeks, and animals were killed eight weeks thereafter (at 23 weeks). All of the animals that received NDMA and were infected developed cholangiocarcinoma and cholangiofibrosis. No such tumour was observed in the group that received either NDMA or parasite alone [$p < 0.001$; Fisher exact test], although cholangiofibrosis was found in some NDMA-treated animals (Thamavit *et al.*, 1978).

A total of 130 male Syrian golden hamsters, six to eight weeks of age, were divided into three groups: 50 animals were infected with 50 *O. viverrini* metacercariae by intragastric intubation, followed 41 days later by a single oral dose of 1.6 mg NDMA; 30 animals received a single oral dose of 1.6 mg NDMA on day 41; and 50 animals were infected with 50 *O. viverrini* metacercariae. Animals were maintained for 70 weeks or were killed when moribund. Cholangiocarcinomas developed in 5/50 infected animals given NDMA at latent periods of 18, 21, 29 (two animals) and 42 weeks after NDMA treatment. No malignant bile-duct tumour was found in any of the hamsters given either NDMA or metacercariae alone, but benign cystic cholangiomas [numbers not specified] were found commonly in these animals (Flavell & Lucas, 1982). [The Working Group noted that the authors did not report cholangiofibrosis in any of the groups. They also noted the single treatment and small dose of the carcinogen.]

A total of 176 male Syrian golden hamsters, six to eight weeks of age, were divided into four groups: 50 animals were infected with 50 *O. viverrini* metacercariae by intragastric intubation, followed 41 days later by a single oral dose of 1.6 mg NDMA; 46 animals received a single oral dose of 1.6 mg NDMA, followed 96 h later by infection with 50 *O. viverrini* metacercariae; 30 animals received a single oral dose of 1.6 mg NDMA; and 50 animals were infected with 50 *O. viverrini* metacercariae. Animals were killed when in poor condition or at the end of the 490-day experimental period. Mortality was highest in infected animals that received NDMA. Cholangiocarcinomas were observed in 5/50 animals (10%) that were first infected and then received NDMA and in 9/46 animals (20%) that received NDMA and were then infected. The difference between these two groups was not significant [Fisher's exact test]. None of the animals given NDMA alone or only infected with parasites developed malignant bile-duct tumours. The mean tumour latency was 249 days (range, 124–346 days) for the group that was first infected and then received NDMA, and that for the group that first received NDMA and were then infected was 308 days (range, 184–393 days); the difference was not significant. Tumours were most frequently found in the right liver lobe, the lobe in the hamster that also contains the largest proportion of *O. viverrini* worms (Flavell & Lucas, 1983).

A total of 280 male Syrian golden hamsters, three to four weeks of age, were divided into four main groups: one remained untreated; others were infected with 12, 25, 50 or 100 *O. viverrini* metacercariae by intragastric intubation; further groups were administered NDMA at 3, 6 or 12 mg/L in the drinking-water at four to five weeks of age for 10 weeks; and others wre infected with 12, 25, 50 or 100 metacercariae two weeks before administration of NDMA at 3, 6 or 12 mg/L in the drinking-water for 10 weeks. All animals were then maintained on basal diet until the end of the experiment at week 40, at which time they were killed. Only 2/17 animals (12%) in the group that received NDMA at 12 mg/L had detectable cholangiocarcinomas. No neoplastic lesion was seen in those that received NDMA at 6 mg/L or 3 mg/L, in those only infected or in untreated controls. In contrast, significant increases in the incidence of cholangiocarcinomas were seen in animals given both NDMA and metacercariae: 14/15, 10/17, 13/19, 7/10 [$p < 0.01$; Fisher's exact test]; and cholangiofibrotic lesions were observed (Thamavit *et al.*, 1987a).

Nitrite and aminopyrine can form NDMA in the stomach under certain conditions. A total of 150 male Syrian hamsters, three to four weeks of age, were divided into eight groups: one group was untreated; a second received 0.1% sodium nitrite in the drinking-water; one received 0.1% aminopyrine in the drinking-water; one received sodium nitrite and aminopyrine in the drinking-water; one was infected with 100 *O. viverrini* metacercariae by a single intragastric intubation; one was similarly infected and four weeks later received sodium nitrite in the drinking-water for 8 or 10 weeks; one was infected and four weeks later received aminopyrine in the drinking-water for 8 or 10 weeks; and the last was infected and four weeks later received sodium nitrite and aminopyrine in the drinking-water for 8 or 10 weeks. Hamsters that received the eight-week drinking-water treatment were killed 12 weeks later, and animals that received the treatment for 10 weeks were killed 20 weeks later. Combined administration of nitrite and aminopyrine for 8–10 weeks resulted in development of two hepatocellular nodules, seven cholangiofibrotic lesions and three cholangiocellular carcinomas. Prior infection with *O. viverrini* metacercariae induced inflammatory and proliferative changes in the livers of infected hamsters and was associated with a significant increase in the incidences of hepatocellular nodules (8; $p < 0.05$), cholangiofibrosis (18; $p < 0.05$) and cholangiocarcinomas (14; $p < 0.01$) (Thamavit *et al.*, 1988a).

A total of 105 male Syrian hamsters, six to eight weeks of age, were divided into four groups: 50 animals received a single intraperitoneal injection of 20 mg/kg bw NDMA, followed 19 days later by infection with 80 *O. viverrini* metacercariae by single intragastric intubation; 25 animals received the intraperitoneal dose of NDMA only; 15 animals were infected with *O. viverrini* only; and 15 animals served as untreated controls. Hamsters were killed when they became moribund or at the end of the experiment at 45 weeks. Among the 43 animals treated with both NDMA and *O. viverrini*, 19 developed cholangiocarcinomas, 40 developed cholangiofibrosis, 15 developed mucinous cystadenomas, 2 developed hepatocellular carcinomas and 42 developed hepatocellular nodules. Although 17/20 (85%) of the hamsters treated with NDMA alone developed hepatocellular nodules, with an average of 3.0 nodules per animal, there was an average of 9.5 nodules per animal in the combined treatment group. No lesion was observed in untreated controls, and 2/15 animals only infected with the parasite developed cholangiofibrosis. The difference in incidence of

cholangiocarcinomas between the combined group (19/45) and the group only infected with *O. viverrini* (0/20) was significant ($p < 0.001$; Fisher's exact test) (Thamavit *et al.*, 1994).

3.2.2 N-*Nitrosodiethylamine*

Hamster: A total of 180 female Syrian hamsters, three to four weeks of age, were divided into eight groups: 20 animals served as untreated controls; 20 animals were infected by gastric intubation with 60 *O. viverrini* metacercariae only; groups of 20–30 animals were infected with 60 *O. viverrini* metacercariae, followed four weeks later by administration of 10, 20 or 40 mg/L *N*-nitrosodiethylamine (NDEA) in the drinking-water for 12 weeks; and groups of 20–25 animals were administered only 10, 20 or 40 mg/L NDEA in the drinking-water for 12 weeks. The animals were killed at week 32. Infection with 60 metacercariae four weeks before administration of 20 or 40 mg/L NDEA resulted in significantly ($p < 0.01$) increased incidences of hepatocellular nodules in the groups also receiving NDEA (12/19 and 23/25, with 2.5 and 7.1 nodules/animal) when compared with the groups that received NDEA alone (3/19 and 9/21 with 0.2 and 0.9 nodules/animal). A high incidence of cholangiofibrosis was seen in animals receiving the combined treatment (Thamavit *et al.*, 1987b).

In a further study, 95 female Syrian golden hamsters, six to eight weeks of age, were divided into five groups: a group of 20 animals received a single intraperitoneal injection of 150 mg/kg bw NDEA dissolved in saline, and two groups of 20 animals each received NDEA followed 18 days later by infection with 50 or 100 *O. viverrini* metacercariae by intragastric intubation; 20 animals received 100 metacercariae without prior treatment with NDEA, and 15 animals were untreated. The animals were killed at the end of week 41. Infection with either 50 or 100 metacercariae of *O. viverrini* after NDEA injection resulted in significantly ($p < 0.01$) enhanced incidences of hepatocellular nodules/animal: 4.3 and 6.8 *versus* 1.4 in animals treated with NDEA alone (Thamavit *et al.*, 1992a).

3.2.3 N-*Nitrosodihydroxydi*-n-*propylamine*

Hamster: A total of 75 male Syrian golden hamsters, three to four weeks of age, were divided into four groups: 25 animals were infected with 100 metacercariae of *O. viverrini* per animal by gastric intubation and two and four weeks later received intraperitoneal injections of 1000 mg/kg bw *N*-nitrosodihydroxydi-*n*-propylamine (NDHDPA); 20 animals were treated with NDHDPA alone; 15 animals were infected with *O. viverrini* alone; and 15 animals served as untreated controls. Animals were killed at week 22. In the group treated only with NDHDPA, 2/20 animals had basophilic hepatocellular foci. Among 19 animals receiving combined treatment with NDHDPA and *O. viverrini*, six developed cholangiocarcinomas [$p = 0.02$], 18 developed cholangiofibrosis [$p = 0.001$] and nine developed hepatocellular nodules [$p = 0.002$] [all Fisher's exact test]; all 19 had hepatocellular basophilic foci, and eight had atypical proliferation of the pancreatic duct. Two of 20 animals given NDHDPA alone had hepatocellular basophilic foci (Thamavit *et al.*, 1988b).

A total of 100 male Syrian hamsters, three to four weeks of age, were divided into four groups: 10 animals served as untreated controls; 20 animals were infected with 80 *O. viverrini* metacercariae by intragastric intubation; 30 animals received three intraperitoneal

injections of 500 mg/kg bw NDHDPA at weeks 16, 17 and 18; and 40 animals were infected with 80 *O. viverrini* and received similar NDHDPA treatment. Animals were maintained on basal diet until they were killed, at week 52, when they were examined histologically. Cholangiocarcinomas occurred in 8/16 animals in the combined treatment group and 0/16 in that receiving NDHDPA alone [$p = 0.001$; Fisher's exact test]. Liver foci were seen in 16/16 hamsters in the combined treatment group and in 14/16 of those given NDHDPA, but the group receiving the combined treatment had a significantly increased number of foci per cm^2 (23.4 ± 7.5 *versus* 3.5 ± 2.6; $p < 0.001$) (Moore *et al.*, 1991).

3.3 Infection with *Opisthorchis viverrini* in combination with administration of other modifying factors

Hamster: A total of 115 male Syrian golden hamsters, six to eight weeks of age, were divided into four groups: 50 animals received five administrations of 60–80 *O. viverrini* metacercariae by intragastric intubation at weeks 0, 8, 16, 24 and 32 and 300 mg/kg bw praziquantel suspended in corn oil five weeks after the time of each administration; 30 animals were given praziquantel alone; and 20 animals received parasites alone, each by the above schedule; 15 animals served as untreated controls. Many of the animals infected with *O. viverrini* metacercariae became moribund and died (16/50 in the combined group; 8/20 in the group receiving infection alone). Surviving animals were killed at the end of week 40. Of the 34 surviving hamsters that received the combined treatment, one developed a cholangiocarcinoma, seven had cholangiofibrosis and one had a hepatocellular nodule. No such lesions were found in hamsters that received the drug alone, but 6/12 surviving hamsters that received infection alone developed cholangiofibrosis (Thamavit *et al.*, 1992b). [The Working Group noted the high mortality in the groups administered *O. viverrini* and the large total number of metacercariae administered.]

A total of 205 female Syrian golden hamsters, six to eight weeks old, were divided into seven groups of 25–40 animals each: three groups received two intraperitoneal injections of 1000 mg/kg bw NDHDPA dissolved in saline at two-week intervals; two weeks later, they were infected with 60 *O. viverrini* metacercariae by intragastric intubation and, at 4, 12 or 20 weeks, received a single dose of 250 mg/kg bw praziquantel suspended in corn oil by intragastric intubation. Two further groups received NDHDPA and *O. viverrini* by the same schedule, but with no praziquantel. One group received injections of saline at two-week intervals, followed two weeks later by infection with *O. viverrini*; another received the saline injections alone. The animals were maintained on basal diet and killed at the end of week 38. Of infected animals given NDHDPA, 16/16 developed cholangiofibrosis, 8/16 developed cholangiocarcinomas (2/18 in the group treated only with *O. viverrini* [$p = 0.015$; Fisher's exact test]) and 16/16 developed hepatic nodules with a multiplicity of 13.6 nodules/cm^2. Praziquantel administration at 4 or 12 weeks reduced the incidences of cholangiocarcinoma to 4/22 and 6/22, respectively. Praziquantal also reduced the multiplicity but not the incidence of hepatocellular nodules (3.6 nodules/cm^2 and 7.4 nodules/cm^2, respectively), but one animal in each of these groups also had a hepatocellular carcinoma. Cholangiofibrosis occurred in all animals treated with NDHDPA and *O. viverrini* plus praziquantel, except in those treated four weeks after infection, of which only 8/22 had cholangiofibrosis (Thamavit *et al.*, 1993).

3.4 Infection with *Opisthorchis felineus*

No data were available to the Working Group.

3.5 Infection with *Clonorchis sinensis* alone

3.5.1 *Rat*

As part of a combination study (see section 3.6.1), a control group of 25 male Wistar albino rats, 8–10 weeks of age, was administered 50 *Clonorchis sinensis* metacercariae by intragastric intubation. A few hepatic necrotic foci and mild inflammatory cell changes were seen in animals from each group killed at 4, 8, 12, 16, 20, 24 and 28 weeks after infection. Neither bile-duct lesions nor liver tumours were observed (Park, 1989). [The Working Group noted the short duration of the study and the inadequate reporting.]

In a further combination study, a control group of 10 male Fischer 344 rats, six weeks old, were each infected with 60 *C. sinensis* metacercariae by intragastric intubation and killed after 40 weeks. The infected animals developed cholangiocellular lesions, including bile-duct proliferation, periductal inflammation, fibrosis with occasional mucinous metaplasia, particularly at the main duct, and extensive areas of ductular proliferation. No tumour was observed (Jang *et al.*, 1990). [The Working Group noted the short duration of the study.]

3.5.2 *Cat*

Three cases of cholangiocarcinoma associated with *C. sinensis* infection were reported in cats (*Felis catus*) (Hou, 1964). Two of the cases were found at necropsy in two approximately four-year-old, well-developed, well-nourished cats out of a total of 215 obtained at random. The two cats harboured 150 and 200 adult *C. sinensis* in the liver. The third case was also in a four-year-old cat, which was one of 26 infected experimentally by feeding a diet of fish (*Ctenopharyngodon idellus*, *Hypophthalmichthys nobilis* and *Mylopharyngodon aethiops*) flesh containing metacercarial cysts of *C. sinensis* for 28 feedings. The animal died of bronchopneumonia; 105 *C. sinensis* were recovered from the bile ducts. The authors reported that the histopathological features of cholangiocarcinoma in the three cats were similar to those of many forms of bile-duct cancer found in humans infected with *C. sinensis* (Hou, 1956).

3.5.3 *Dog*

Cholangiocarcinoma associated with *C. sinensis* infection was also reported in one well-developed, well-nourished eight-year-old female chow dog, which had suffered from abdominal enlargement for an unknown period before death (Hou, 1965a). The histopathological features of the cholangiocarcinoma were reported to be similar to those of a form of bile-duct cancer found in humans infected with *C. sinensis* (Hou, 1956).

3.6 Infection with *Clonorchis sinensis* in combination with administration of known carcinogens

3.6.1 *Aflatoxin B_1*

A total of 75 male Wistar albino rats, 8–10 weeks old were divided into three groups: 25 rats were fed aflatoxin B_1 at 1 mg/kg diet for 12 weeks; 25 rats were infected by

administration of 50 *C. sinensis* metacercariae by intragastric intubation; and 25 animals were infected with *C. sinensis* and fed aflatoxin B_1 in the diet concomitantly. Three rats from each group were killed at four-week intervals up to 28 weeks after the beginning of treatment. Well-differentiated hepatocellular carcinomas were detected in two of three rats given the combined treatment and alive at 28 weeks; such tumours were not seen in rats treated with aflatoxin B_1 alone and killed at the same intervals (Park, 1989). [The Working Group noted the inadequate reporting of the study and the small comparison groups in the serial killings.]

3.6.2 N-*Nitrosodimethylamine*

Rat: A total of 101 male Fischer 344 rats, six weeks of age, were divided into six groups: 20 animals were each infected with 60 *C. sinensis* metacercariae by single intragastric intubation four weeks before receiving NDMA at 25 mg/L in the drinking-water for eight weeks; 20 animals were infected with *C. sinensis* while receiving NDMA at 25 mg/L in the drinking-water for eight weeks; 20 animals were infected with *C. sinensis* one week after NDMA treatment; 19 animals received NDMA in the drinking-water alone for eight weeks; 10 animals were infected with *C. sinensis* alone; and 12 animals served as untreated controls. The animals were killed at week 40, and all were found to have heavy helminthic loads. Livers were examined immunohistochemically for foci of the placental form of glutathione *S*-transferase. Animals infected before NDMA administration had significantly ($p < 0.05$) increased numbers of foci. No such effect was seen when animals were infected with *C. sinensis* during or after exposure to NDMA (Jang *et al.*, 1990).

Hamster: A total of 48 Syrian golden hamsters [sex unspecified], three to four weeks old, were divided into four groups: 12 animals received NDMA at 15 mg/L in the drinking-water for eight weeks and were given 10 metacercariae of *C. sinensis* suspended in saline by intragastric intubation seven days after the beginning of NDMA administration; 12 animals received the NDMA treatment alone; 12 received the helminthic treatment alone; and 12 animals served as untreated controls. After 11 weeks, 6/8 (75%) infected animals given NDMA developed cholangiocarcinomas, 8/8 developed cholangiofibrosis and 8/8 developed cholangiofibroma. Of the 12 animals given NDMA alone, two developed cholangiofibrosis and cholangiofibroma; of those given the helminth alone, 5/12 developed cholangiofibrosis. No lesions were observed in the 12 untreated controls (Lee *et al.*, 1993).

A total of 90 Syrian golden hamsters [sex unspecified], weighing 50–60 g, were divided into six groups of 15 animals each: one group received NDMA at 15 mg/L in the drinking-water for four weeks, followed one week later by administration of 15 metacercariae of *C. sinensis* suspended in saline by intragastric intubation; five weeks later the animals received oral administrations of 200 mg/kg bw praziquantel daily for three days. Another group was similarly infected with *C. sinensis* metacercariae but was treated with praziquantel for three days before treatment with NDMA. A further group received concomitant administration of NDMA and infection with *C. sinensis*. One control group received NDMA and another was infected with the helminth only. A final group served as untreated controls. At the end of 13 weeks, the group that had received concomitant treatment with NDMA and *C. sinensis* had 11/15 cholangiocarcinomas, 3/15 cholangiofibromas and 1/15 cholangiofibroses. In the group infected one week after NDMA treatment and given praziquantel,

3/15 had cholangiocarcinomas, 3/15 had cholangiofibromas and 6/15 had cholangiofibroses. In the group with combined treatment but given praziquantel three days before NDMA, 11/15 animals developed cholangiofibroses. In the group given NDMA alone, 4/15 animals had cholangiofibroma and 5/15 had cholangiofibroses; and in the group receiving only helminthic infection, 12/15 animals developed cholangiofibroses. No cancerous or pre-cancerous lesion of the bile duct was found in the untreated control group (Lee *et al.*, 1994).

3.6.3 *2-Acetylaminofluorene*

Hamster: Groups of 50 and 60 female Syrian golden hamsters, 8–10 weeks old, received 0 or 40 *C. sinensis* metacercariae per animal orally and were fed diets containing 0.03% 2-acetylaminofluorene for 40 weeks. After this time, all surviving animals were fed normal diets without carcinogen. Small numbers of animals from both groups were killed every three to four weeks from 0 up to 54 weeks, at which time the experiment was terminated. In animals that lived beyond 25 weeks, the incidence of cholangiocarcinomas was significantly ($p < 0.05$) higher in the infected group (11/14 animals) than in the uninfected group (6/17 animals). Metastases to other organs were observed only in infected animals with cholangiocarcinomas. The first bile-duct tumours were noted at 25 weeks in the infected group and at 35 weeks in the uninfected group (Iida, 1985).