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Prenatal toxicity of acyclovir in rats

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Abstract. Pregnant rats were treated during organogenesis with s.c. injections of acyclovir and the embryos were evaluated on day 11.5 of gestation (crown-rump length, somites, protein content, score, abnormalities, histological examination). After eight injections of 50 mg/kg body wt on days 9, 10, and 11 of pregnancy a reduction of the crown-rump length was noticed. After 100 mg/kg this effect was more pronounced. With two or three applications of this dose on day 10 specific embryonic abnormalities were visible: the shape of the head was abnormal, the width of the skull had decreased resembling a beak-like visceral cranium. With a single administration of 200 mg/kg on day 10 we found a similar but slightly more pronounced outcome. A drastic change of all variables was obtained after eight injections of 100 mg/kg on days 9, 10, and 11. Comparatively we measured maternal plasma concentrations of acyclovir 1 h after the administration of 50, 100 or 200 mg/kg body wt. After an injection of 50 mg/kg on days 9, 10, and 11 of gestation (three injections/day) the plasma levels ranged from 19.1 to 40.0 mg/l (1 mg/l = 4.44 μM). No cumulation was observed. In contrast, a cumulative effect was detected following a dose of 100 mg/kg. After the first injection of this dose a mean value (± SD) of 60.3 ± 14.7 mg/l (n = 16) was obtained. In this case a third injection increased the mean plasma level to 124.6 ± 16.6 mg/l (n = 5). Further injections, however, led to decreasing levels. One hour after administration of 200 mg/kg body wt acyclovir levels ranged from 120.0 to 163.9 mg/l. We conclude that acyclovir, at doses leading to plasma concentrations well above the therapeutic level in the dam, interferes with the embryonic development in the rat. Acyclovir induces typical gross structural abnormalities which have been first observed using a whole embryo culture system.

Key words: Acyclovir – Prenatal toxicity – Rat – Virustatics

Introduction

Many derivatives of naturally occurring nucleosides have been investigated for their cytostatic or antiviral activity. Replacing the cyclic sugar of deoxyguanosine by a linear side-chain led to acyclovir, 9-(2-hydroxyethoxymethyl)guanine, a nucleoside analogue which combines high activity against herpes viruses with a remarkably low toxicity for uninfected mammalian cells (Elion 1982).

In routinely performed studies on the reproductive toxicity of the nucleoside analog no teratogenic potential was found with doses up to 2 × 25 mg/kg s.c. daily from day 6 to day 15 of gestation in the rat (Moore et al. 1983). However, with a rat whole-embryo culture technique Klug et al. (1985a) showed that acyclovir is able to induce abnormal development in vitro. At concentrations of 25 μM (= 5.6 mg/l) in the culture medium impaired development of the ear anlagen was noticed. With increasing concentrations (50, 100, 200 μM) additional disturbances of embryonic differentiation were demonstrated in vitro. The drug induced gross structural abnormalities, especially of the telencephalon.

The doses tested in vivo were restricted by the finding that acyclovir causes nephrotoxic effects due to poor water solubility and crystallization of acyclovir within the tubular system of the kidneys (Tucker et al. 1983). Only with the background of the results from the in vitro experiments is it reasonable to test higher doses in vivo over a restricted period of time.

We performed some preliminary studies in rats on the prenatal toxicity of acyclovir to compare the in vivo toxicity with the effects observed with the whole-embryo culture: in these investigations the dams were sacrificed on day 11.5 of pregnancy and the embryos were evaluated in exactly the same manner as at the end of a whole-embryo culture period, described by Klug et al. (1985b). Brief presentations of the results obtained have been given before (Neubert et al. 1986; Stahlmann and Chahoud 1987).

For a rational evaluation of the drug effects in vivo, knowledge of the pharmacokinetics of the substance is essential. Therefore, we conducted extended pharmacokinetic studies of acyclovir in pregnant rats and, furthermore, took single or multiple blood samples during the experiments described here from the tail vein of the dams 1 h after treatment (Bochert et al. 1987).

Materials and methods

Animal maintainance and mating procedure. Wistar rats (Bor: Wisw/SPF, TNO; Fa. Winkelmann, Borchen, FRG) were kept under spf conditions at a constant day/night cycle (light: 9.00–21.00 hours). The animals were fed Altromin-1324 and received tap water ad lib. One male was

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caged with three females for 2 h (6.00–8.00 a.m.) and the first 24 h period following the mating procedure was called "day 0" of pregnancy if sperms were detected in the vaginal smears.

Drug application and blood sampling. Acyclovir was used as the commercially available preparation [Zovirax, Deutsche Wellcome, Burgwedel, FRG]. The vials were dissolved in 12.5 ml distilled water to obtain a 2% solution. In all experiments the drug was injected subcutaneously at 7.00 and/or 12.00 and/or 17.00 hours on day 9 and/or 10 of pregnancy. Since the section was performed on day 11.5 at 16.00–17.00 hours, treatment on day 11 was limited to the two early administrations. The volume of injection was 10 ml/kg body wt for the 200 mg/kg dose and 5 ml/kg and 2.5 ml/kg for the 100 mg/kg and 50 mg/kg doses, respectively.

Blood samples were collected from a tail vein after warming the tail in ca. 56° C water using cannulae without adaptors as described by Furuhama and Onodera (1983). Blood was collected in heparinized haematocrit capillaries (four capillaries of 75 µl per sample) which were centrifuged immediately. The capillaries were cut to collect the plasma which was then frozen at −25° C until it was analyzed.

As we learned from separately conducted experiments, the peak level of the drug under the conditions of these experiments occurs approximately 45–90 min after injection. In the experiments described here we usually took blood samples 1 h after injection ("peak level"). In some treatment groups with multiple injections, samples for a "trough level" (immediately before injection) were obtained as well.

On the afternoon of day 11 of pregnancy the animals were sacrificed and blood samples were obtained by decapitation.

Plasma analysis. Plasma samples were analyzed by HPLC following deproteinization with TFA using a reversed phase ion-pairing HPLC method (Smith and Walker 1985).

Evaluation of embryos. All embryos were evaluated on day 11.5 of gestation (section time between 16.00 and 17.00 hours), corresponding to the time of evaluation after a whole embryo culture time of 48 h starting on day 9.5. The conceptuses were removed from the uterus and transferred into plastic petri dishes containing 0.9% NaCl solution. With the aid of a dissecting stereomicroscope, the crown-rump length was measured using an ocularimeter and the number of somites was counted. Additionally, the development was evaluated by means of a scoring system which has been established in our laboratory and has recently been described in detail (Klug et al. 1985b). The more advanced the development of the embryo the higher the score. The appearance of structural abnormalities was also considered. At least 20–30% of the embryos from each treatment group were histologically examined. All embryos which were not histologically examined were used for determination of the protein content. Each embryo was homogenized separately in 10% NaOH solution, the final volume being 200 µl. The protein content was measured using an automatic bichromatic analyzer (ABA 100, Fa. Abbott) with the biuret method.

After evaluation with the scoring system the data obtained were entered into the computer (PDP 11-34/VAX.) and stored. A statistical evaluation was performed using the Minitab programme (Minitab Inc., Univ. Park, Pennsylvania, USA). The data were statistically analyzed by means of the Mann-Whitney test.

Histological examination. For the light microscopic investigations the material was fixed in Bouin's solution, embedded in paraffin and stained with hematoxylin-eosin, Azan or PAS.

Results

1. Evaluation of embryos on day 11.5 of development

Table 1 gives the median values and the first and third quartiles of the variables evaluated (crown-rump length, somites, protein content and score) of all embryos. The percentage of "abnormal" embryos is also given. The me-

Table 1. Prenatal toxicity of acyclovir in rats. Pregnant rats were treated as indicated (day 9: 7.00; 12.00 and 17.00 hours; day 10: 7.00, 12.00 and 17.00 hours; day 11: 7.00 and 12.00 hours by s.c. injection of acyclovir or 0.1 *N*-NaOH (vehicle control). All embryos were evaluated on day 11.5 of gestation in exactly the same way as the embryos are routinely evaluated at the end of the whole-embryo culture period (for a detailed description of the method cf. KLUG et al. 1985b)

Dose (s.c.)	Day of gestation	Evaluation of embryos (day 11.5)[b]					
		n	CR	Somites	Protein	Score	Abnormal (%)
1) 3 × 0.1 *N*-NaOH[a]	10	22	4.02 (3.82/4.20)	26 (25/26.5)	299 (244/353)	40 (40/40)	–
2) 8 × 50 mg AC/kg	9, 10, 11	32	3.63 (3.49/3.76)**	26 (25/27)	–	40 (39/40)	3
3) 1 × 100 mg AC/kg	10	68	3.54 (3.36/3.72)**	25 (24/26)*	226 (203/272)**	40 (38/40)	1.5
4) 2 × 100 mg AC/kg	10	95	3.48 (3.24/3.66)**	25 (24/26)**	212 (180/244)**	37 (35/37)**	75
5) 3 × 100 mg AC/kg	10	31	3.12 (2.88/3.18)**	24 (20/25)**	140 (86/166)**	34 (32/35)**	100
6) 1 × 200 mg AC/kg	10	51	3.00 (2.76/3.12)**	24 (22/24)**	129 (101/153)**	34 (32/35)**	100
7) 8 × 100 mg AC/kg	9, 10, 11	23	1.80 (1.50/2.40)**	12 (11/15)**	–	25 (23/28)**	100

[a] Vehicle control; dose: 5 ml/kg

[b] Median values and 1st and 3rd quartile are given in this table; *n* = number of embryos evaluated; *CR* = crown-rump length (mm); Somites = number of somite pairs; Protein = protein content (µg/embryo); for "Score" cf. Klug et al. (1985b)

* $p \leq 0.05 > 0.01$ compared with NaOH control

** $p \leq 0.01$ compared with NaOH control

Table 2. Maternal plasma concentrations of acyclovir (mg/l) and evaluation of embryos on day 11.5 of pregnancy after administration of eight injections of acyclovir (50 mg/kg body wt) to the dam on days 9–11 of pregnancy*

Animal no.	Plasma concentration (mg/l)				Evaluation of embryos (day 11.5)**					
	Day 9 18.00	Day 10 18.00	Day 11 13.00	Section***	*n*	*CR*	Somites	Protein	Score	Abnormal (%)
1	25.1	19.1	21.4	1.1	10	3.63	26.0	–	40	10
2	40.0	35.1	21.5	3.6	9	3.53	26.0	–	40	–
3	25.8	20.1	33.5	1.6	13	3.84	27.0	–	40	–

* Application time: Day 9: 7.00, 12.00, 17.00; day 10: 7.00, 12.00, 17.00; day 11: 7.00, 12.00
** Median values are given in this table; *n* = number of embryos evaluated; *CR* = crown-rump length (mm); Somites = number of somite pairs; Protein = protein content (μg/embryo); for "Score" cf. Klug et al. (1985b)
*** Section was performed on day 11 at 16.45 (1), 17.10 (2) and 17.30 (3)

dian values of the embryos of each dam are given in Tables 2–7, which also show the drug levels of the mother animals after injection.

The crown-rump length seems to be the most sensitive parameter influenced by acyclovir. After 8 × 50 mg/kg from day 9 to day 11 of gestation a significant decrease in crown-rump length was observed (Table 2). Somite number and score were not significantly altered; the protein content was not determined in this group, since all embryos were histologically investigated. Only one embryo out of 32 showed abnormalities. This may not be drug related. Figure 1b gives an example of a typical embryo of this group. Macroscopically it very closely resembles the NaOH-treated controls (Fig. 1a).

After a single injection of 100 mg/kg on day 10 the crown-rump length and the protein content were decreased in comparison with the NaOH-treated controls. No major differences were detectable with respect to the time of the day on which the drug was injected (7.00, 12.00 or 17.00 hours). With this dose only one out of 68 embryos showed abnormalities (Table 3).

There are clear differences in the embryonic development between the embryos shown in Table 3 (1 × 100 mg/kg at different times of the day) and Table 4 (2 × 100 mg/kg at different times of the day). With this later administration regime the score is lower and the frequency of abnormalities is drastically elevated. A closer analysis of these groups shows that the litters were either almost completely affected or showed very little effect. Two out of ten litters had abnormality rates below 10% (no 4 and 5, Table 4); this means that two out of 23 embryos were malformed. On the other hand, only three of the remaining 72 embryos showed no abnormalities.

Three doses of 100 mg/kg on day 10 of gestation induced specific defects in the cranial region of the rat embryos which very closely resembled the morphological outcome of the in vitro cultured embryos with an acyclovir concentration of 100 μM (= 22.5 mg/l) in the culture medium (Fig. 2). The shape of the head was abnormal: the width of the skull had decreased, resembling a beak-like visceral cranium (Fig. 1e). All embryos of three rats show abnormalities, the median values of all variables followed are significantly altered. The most drastic change concerns the protein content. Multiple blood samples could be obtained from only two dams (Table 5).

Similar results can be obtained by a single application of 200 mg/kg on day 10 (Table 6, Fig. 1d). Again, no significant differences were observed with respect to the time of day (7.00, 12.00 or 17.00 hours) when the substance was injected.

Treatment of the mother animals with eight injections from day 9 to 11 of gestation resulted in most severely affected embryos (Table 7, Fig. 1f). The median values of all variables tested were drastically decreased. The typical changes in the head region obtained with 3 × 100 mg/kg and 1 × 200 mg/kg were visible in this group too and were far more pronounced.

2. Plasma concentrations of acyclovir

From 32 pregnant rats treated with different doses of acyclovir we analyzed a total of 64 plasma samples to monitor drug levels during treatment. The values are given as mg/l (1 mg/l = 4.44 μM).

One hour after injection of 50 mg/kg body wt plasma concentrations ranged from 19.1 to 40.0 mg/l (Table 2). No significant change in plasma concentrations occurred after multiple dosing.

A total of 16 samples from different treatment groups was analyzed 1 h after the first injection of 100 mg/kg body wt on day 10 of gestation. The mean value ± standard deviation was calculated as 60.3 ± 14.7 mg/l (Fig. 3). Drug levels were significantly higher after the third injection of the same dose within a time period of 10 h. Under these conditions the mean value was 124.6 ± 16.6 mg/l (*n* = 5). This cumulative effect diminished over longer treatment periods. Table 7 gives the plasma concentrations 1 h after the third, the sixth and the eighth injection of 100 mg/kg in three rats. A decrease in concentration from 126.1 ± 23.3 to 95.5 ± 17.2 and 79.3 ± 22.7 mg/l (mean values ± SD) is obvious.

The highest dose tested for embryotoxic effects was 200 mg/kg body wt (Table 6; Fig. 3). Drug levels ranged from 120.0 to 163.9 mg/l 1 h after injection (mean value ± SD: 143.4 ± 17.1 mg/l).

3. Histological evaluation of the embryos

In general, the histological examination confirmed the findings from the macroscopical evaluation: on a microscopical level, too, the embryonic impairment closely resembled the type of changes observed after in vitro cultivation of the embryos. Figure 4 shows sagittal sections of two rat embryos after prenatal exposure to acyclovir in com-

parison to a control embryo. The crown-rump length reduction and the morphological changes in the head region are obvious in the embryos after prenatal treatment of the dams on day 10 of pregnancy with two injections of 100 mg/kg or even more pronounced after treatment with a single injection of 200 mg/kg. The effects of this dosage on the brain are shown in Fig. 5 at a higher magnification. A distinct decrease of skull and brain anlagen is recognizable in comparison to controls. Numerous necroses are visible on the sagittal section in the neuroepithelium of the

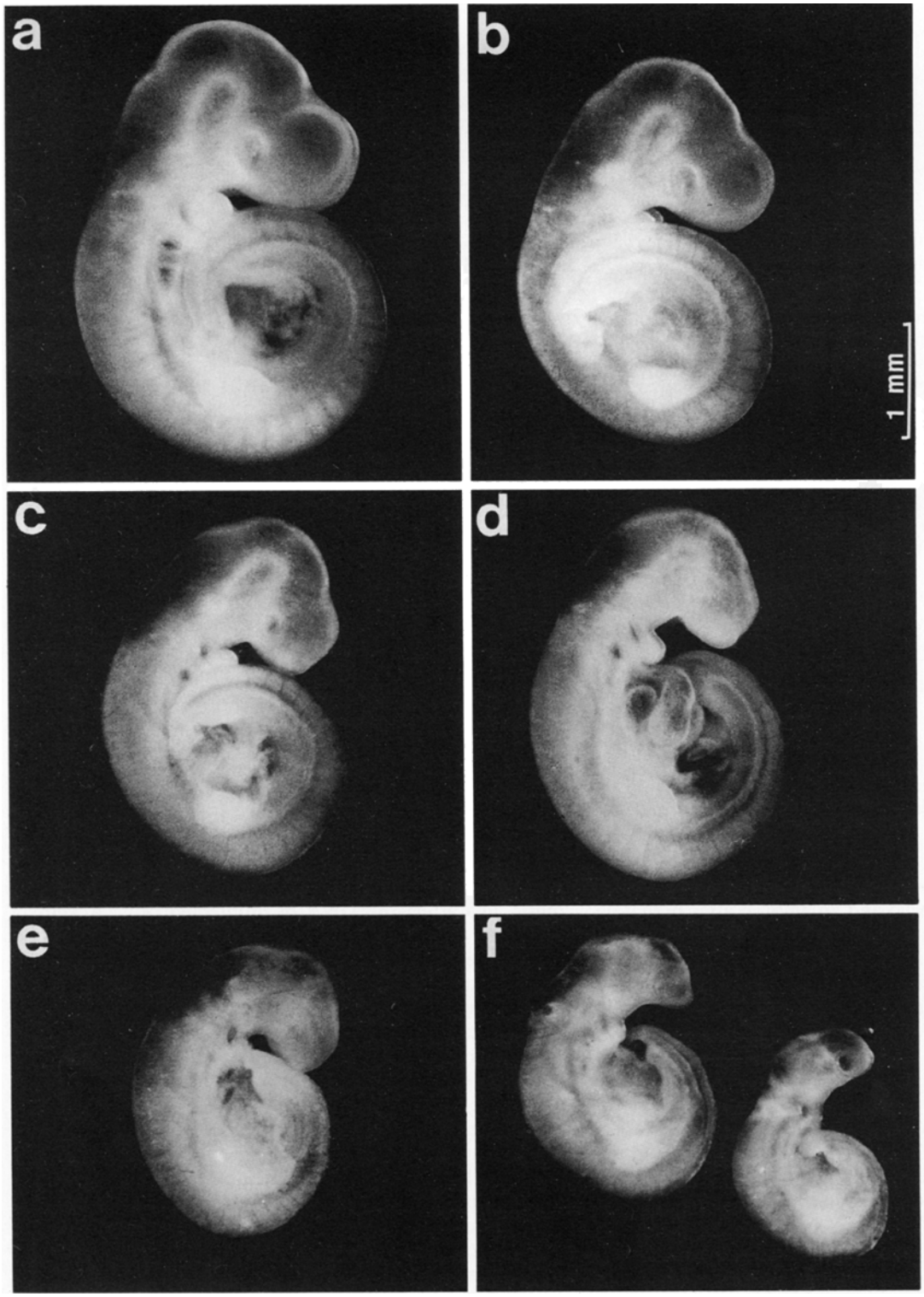


Fig. 1a–f. 11.5-day-old rat embryos after maternal treatment with **a** 3 × 0.1 N NaOH (5 ml/kg) on day 10 of gestation (control); **b** 8 × acyclovir (50 mg/kg body wt) from day 9 (7:00 a.m.) to day 11 (12:00) of gestation; **c** 2 × acyclovir (100 mg/kg body wt) on day 10 (7:00 and 12:00) of gestation; **d** 1 × acyclovir (200 mg/kg body wt) on day 10 (7:00) of gestation; **e** 3 × acyclovir (100 mg/kg body wt) on day 10 (7:00, 12:00, 17:00) of gestation; **f** 8 × acyclovir (100 mg/kg body wt) from day 9 (7:00) to day 11 (12:00) of gestation

Table 3. Maternal plasma concentrations of acyclovir (mg/l) 1 h after dosing and evaluation of embryos on day 11.5 of pregnancy after administration of a single s.c. injection of acyclovir (100 mg/kg body wt) to the dam at 7.00 or 12.00 or 17.00 hours on day 10 of pregnancy

Animal no.	Application time			Plasma concentration (mg/l)			Evaluation of embryos (day 11.5)*					
	Day 10			Day 10			*n*	*CR*	Somites	Protein	Score	Abnormal (%)
	7.00	12.00	17.00	8.00	13.00	18.00						
1	x	–	–	53.7	–	–	9	3.54	26	213	40	0
2	x	–	–	50.7	–	–	12	3.75	25.5	288	40	0
3	–	x	–	–	76.7	–	11	3.36	25	234	39	0
4	–	x	–	–	53.8	–	10	3.69	26	263	40	0
5	–	–	x	–	–	57.8	6	2.91	22.5	126	34	16
6	–	–	x	–	–	59.4	12	3.51	25.5	221	40	0
7	–	–	x	–	–	80.1	8	3.37	25.0	225	37.5	0

* Median values are given in this table; *n* = number of embryos evaluated; *CR* = crown-rump length (mm); Somites = number of somite pairs; Protein = protein content (µg/embryo); for "Score" cf. Klug et al. (1985b)

Table 4. Maternal plasma concentrations of acyclovir (mg/l) 1 h after dosing and evaluation of embryos on day 11.5 of pregnancy after administration of two s.c. injections of acyclovir (100 mg/kg body wt) to the dam at 7.00 and 12.00 or 7.00 and 17.00 or 12.00 and 17.00 hours on day 10 of pregnancy

Animal no.	Application time			Plasma concentration (mg/l)			Evaluation of embryos (day 11.5)*					
	Day 10			Day 10			*n*	*CR*	Somites	Protein	Score	Abnormal (%)
	7.00	12.00	17.00	8.00	13.00	18.00						
1	x	x	–	63.2	–	–	8	3.06	24	198	35.5	100
2	x	x	–	49.3	155.6	–	9	2.76	22	140	33	100
3	x	x	–	49.9	–	–	8	3.60	26	219	36.5	100
4	x	x	–	63.8	–	–	11	3.60	25	249	39	9
5	x	–	x	55.7	–	79.0	12	3.60	26	237	40	8
6	x	–	x	31.9	–	72.1	10	3.21	23.5	169	35.5	80
7	x	–	x	–	–	–	8	3.36	25	212	37	100
8	x	–	x	–	–	–	9	3.66	26	225	37	88
9	–	x	x	–	–	133.0	11	3.60	26	232	36	100
10	–	x	x	–	–	131.9	9	3.66	26	243	37	100

* Median values are given in this table; *n* = number of embryos evaluated; *CR* = crown-rump length (mm); Somites = number of somite pairs; Protein = protein content (µg/embryo); for "Score" cf. Klug et al. (1985b)

prosencephalon and mesencephalon. In Fig. 6 two cross-sections of a control embryo and an embryo after prenatal treatment of the dam with a single injection of 200 mg/kg on day 10 are given. After exposure to the nucleoside analogue the diencephalon anlage is flattened, the neuroepithelium is flattened, and no eye cups are formed. Some details are shown at higher magnification on Fig. 7.

Discussion

Within a few years acyclovir has become the most frequently used drug for infections due to herpes and related viruses (Hayden and Douglas 1985). Its therapeutic efficacy and superiority to other drugs has been proven in well-conducted clinical trials for herpes encephalitis (Whitley et al. 1986) and some other types of infections – especially in immuno-compromised hosts (Shepp et al. 1986; Andersson et al. 1986). The introduction of this drug must be considered a major therapeutic improvement, although acyclovir does not have an effect on the relapse rate of herpes infections.

All drugs known so far which inhibit viral DNA synthesis also affect the DNA of uninfected cells. Acyclovir shows an unusually high degree of selectivity: its conversion to the monophosphate is catalyzed with a high degree of specificity by the viral thymidine kinase. However, it is well known that at high concentrations an effect on the DNA metabolism of uninfected cells also occurs.

The first important prerequisite for an effect of acyclovir on DNA is its conversion to the corresponding monophosphate. This step is catalyzed preferentially by the viral thymidine kinase. The monophosphate is subsequently converted to acyclovir triphosphate by cellular enzymes. Cellular thymidine kinase does not catalyze the critical first phosphorylation step, although other yet unidentified enzymes are capable of this reaction to a small extent. The amounts of the biologically active acyclovir triphosphate formed in herpes virus infected cells is 40–100 times greater than in uninfected vero cells (Elion 1982).

Klug et al. (1985a) described abnormal rat embryos with concentrations of acyclovir in the whole-embryo culture system at 50 µM (11.3 mg/l) and higher. These find-

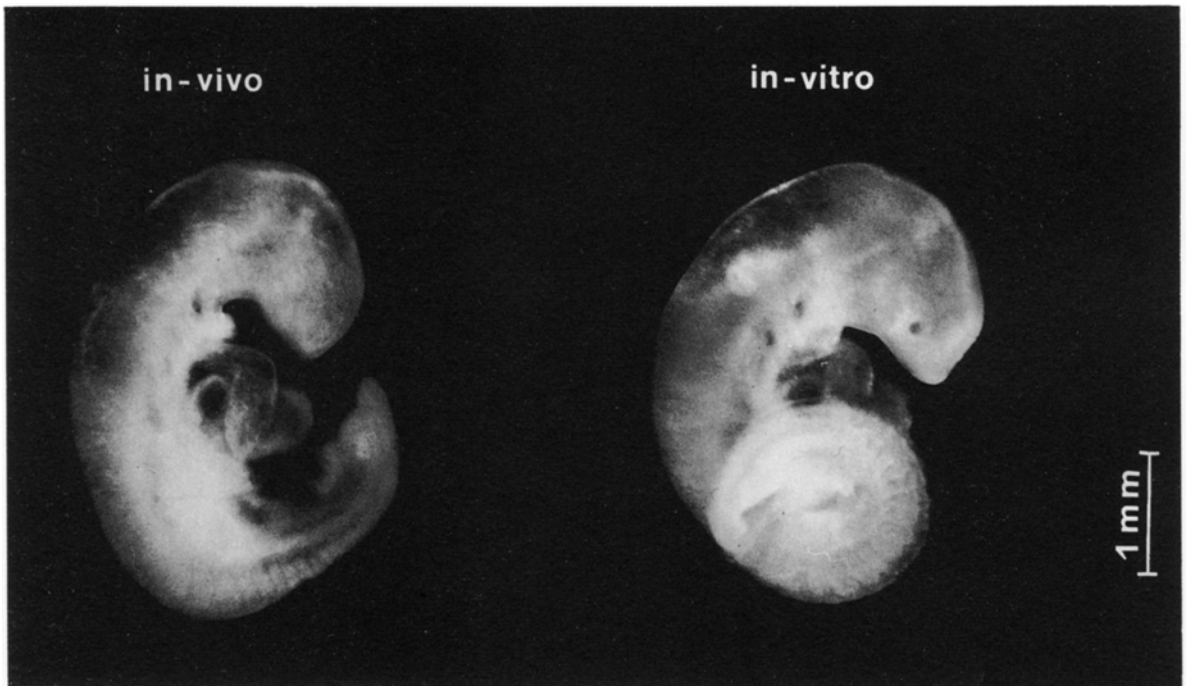


Fig. 2a, b. Morphological outcome of rat embryos after exposure to acyclovir under in vitro and in vivo conditions. **a** Embryo from in vivo study; three s.c. injections of 100 mg/kg body wt on day 10 of gestation (7:00, 12:00, and 17:00); evaluation on day 11.5. **b** Embryo from whole-embryo culture; culture period day 9.5 to 11.5 of development; acyclovir concentration in the culture medium: 100 μM (= 22.5 mg/l)

Table 5. Maternal plasma concentrations of acyclovir (mg/l) 1 h after dosing and immediately before dosing and evaluation of embryos on day 11.5 of pregnancy after administration of three s.c. injections of acyclovir (100 mg/kg body wt) to the dam at 7.00, 12.00 and 17.00 hours on day 10 of pregnancy

Animal no.	Application time			Plasma concentration (mg/l)					Evaluation of embryos (day 11.5)*					
	Day 10			Day 10					*n*	*CR*	Somites	Protein	Score	Abnormal (%)
	7.00	12.00	17.00	8.00	12.00	13.00	17.00	18.00						
1	x	x	x	49.2	26.4	104.1	34.4	124.0	12	3.21	24.5	162	34.5	100
2	x	x	x	87.8	–	–	–	–	9	2.88	20	62	31	100
3	x	x	x	81.1	19.4	121.4	18.3	120.7	10	3.09	24	141	34	100

* Median values are given in this table; *n* = number of embryos evaluated; *CR* = crown-rump length (mm); Somites = number of somite pairs; Protein = protein content (μg/embryo); for "Score" cf. Klug et al. (1985b)

Table 6. Maternal plasma concentrations of acyclovir (mg/l) 1 h after dosing and at the time of the section and evaluation of embryos on day 11.5 of pregnancy after administration of a single s.c. injection of acyclovir (200 mg/kg body wt) to the dam at 7.00 or 12.00 or 17.00 hours on day 10 of pregnancy

Animal no.	Application time			Plasma concentration (mg/l)				Evaluation of embryos (day 11.5)*					
	Day 10			Day 10				*n*	*CR*	Somites	Protein	Score	Abnormal (%)
	7.00	12.00	17.00	8.00	13.00	18.00	Section**						
1	x	–	–	120.0	–	–	0.5	7	3.00	24	138	34	100
2	x	–	–	135.4	–	–	<0.5	6	2.91	24	130	33	100
3	–	x	–	–	157.0	–	8.8	10	2.70	23	97	32	100
4	–	x	–	–	163.9	–	1.2	8	3.12	24	155	36	100
5	–	–	x	–	–	153.0	–	9	3.06	22	135	34	100
6	–	–	x	–	–	131.0	10.3	11	3.00	24	135	35	100

* Median values are given in this table; *n* = number of embryos evaluated; *CR* = crown-rump length (mm); Somites = number of somite pairs; Protein = protein content (μg/embryo); for "Score" cf. Klug et al. (1985b)
** Section was performed on day 11 at 15.30 (1), 17.30 (2), 16.00 (3), 17.00 (4), 16.20 (5), 16.40 (6)

Table 7. Maternal plasma concentrations of acyclovir (mg/l) 1 h after the last daily dosing and at the time of the section and evaluation of embryos on day 11.5 of pregnancy after administration of eight s.c. injections of acyclovir (100 mg/kg body wt) to the dam on days 9–11 of pregnancy*

Animal no.	Plasma concentration (mg/l)				Evaluation of embryos (day 11.5)**					
	Day 9 18.00	Day 10 18.00	Day 11 13.00	Section**	*n*	*CR*	Somites	Protein	Score	Abnormal (%)
1	125.4	79.6	67.5	25.3	7	1.56	12	–	24	100
2	103.1	93.1	64.9	24.5	10	2.43	14	–	28	100
3	149.7	113.8	105.4	28.0	6	1.50	11	–	23	100

* Application time: Day 9: 7.00, 12.00, 17.00; day 10: 7.00, 12.00, 17.00; day 11: 7.00, 12.00
** Median values are given in this table; *n* = number of embryos evaluated; *CR* = crown-rump length (mm); Somites = number of somite pairs; Protein = protein content (μg/embryo); for "Score" cf. Klug et al. (1985b)
*** Section was performed on day 11 at 16.45 (1), 17.10 (2), 17.30 (3)

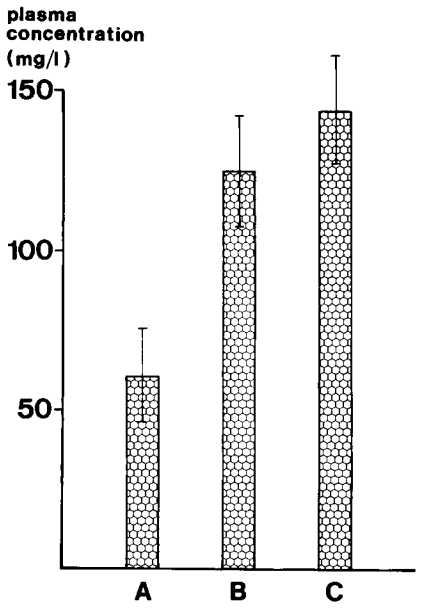


Fig. 3. Plasma concentrations of acyclovir in pregnant rats 1 h after the first or the third s.c. injection of 100 mg/kg body wt on day 9, 10 or 11 of gestation or after the first injection of 200 mg/kg body wt

ings suggest specific teratogenic effects at rather low concentrations which might be explained by a relatively high sensitivity of embryonic structures to the nucleoside analogue. This could be explained with the high proliferation rate and amount of DNA synthesis in embryonic cells.

Conventionally performed teratogenicity studies (segment II studies) are often inappropriate to answer detailed questions on the teratogenic potential of a chemical. Drug administration at high doses throughout the whole period of organogenesis and longer (e.g. days 6–15 in the rat) may induce embryomortality, growth retardation and other effects on embryonic development. Under such circumstances, and without further information, it may be difficult to evaluate an observed embryotoxic effect since it is difficult to distinguish the "specific drug effect" from indirect influences caused by maternal toxicity if both effects occur at similar doses.

Moore et al. (1983) used a conventional protocol and found no signs of prenatal toxicity in rats with acyclovir up to a subcutaneous dose of 2 × 25 mg/kg body wt daily. Under these conditions maternal plasma peak concentrations of about 16 mg/l were measured and no signs of maternal toxic effects were detectable.

At higher doses acyclovir causes crystalline nephropathy ("secondary toxicity"). In order to obtain some information on the embryotoxic potential of the drug it is necessary to apply relatively high doses, which exhibit maternal toxic effects.

Supplementation of whole animal experiments with in vitro methods can help in such a situation to distinguish between specific drug effects and secondary influences caused by maternal toxicity.

Information on the teratogenic potential of acyclovir without maternal influence come from the rat whole-embryo in vitro studies (Klug et al 1985a) and from investigations in the chick embryo (Heinrich-Hirsch and Jacob-Müller 1987). From the clear-cut results with acyclovir in the rat whole-embryo culture system we were able to deduce the period of high susceptibility (day 10) to the acyclovir effect.

Surprisingly, we found a very similar morphological outcome after treatment on day 10 of gestation as had been seen before in the whole-embryo in vitro studies. Macroscopically and histologically the typical CNS defects (telencephalon) are visible. Defects of the head and tail are visible, too, if the evaluation is done on day 21 or even postnatally (Stahlmann and Chahoud 1987). These anomalies will be described in detail in a following paper.

There seems to be a very steep dose-response curve as multiple doses of 50 mg acyclovir/kg during organogenesis induce no defects, but 200 mg/kg on day 10 of gestation as a single injection or divided into two doses on day 10 produce CNS defects. It seems that only with high peak levels is the influence on embryonic cells strong enough to produce impairments that will not be repaired and can be noticed as malformations.

For a closer analysis of the effects observed in the in vitro and in our in vivo studies it is important to look at the data existing on acyclovir concentrations in rats, especially in pregnant rats and rat fetuses.

De Miranda and coworkers (1981) conducted pharmacokinetic and metabolic studies with radiolabelled acy-

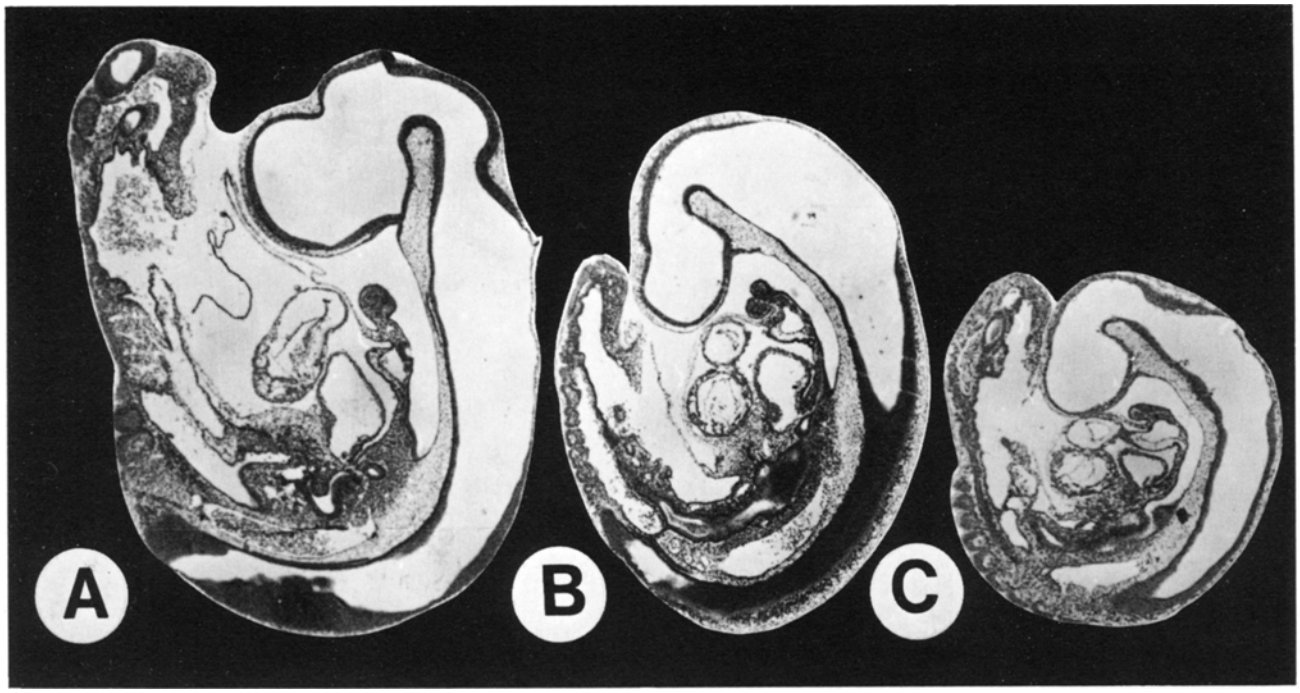
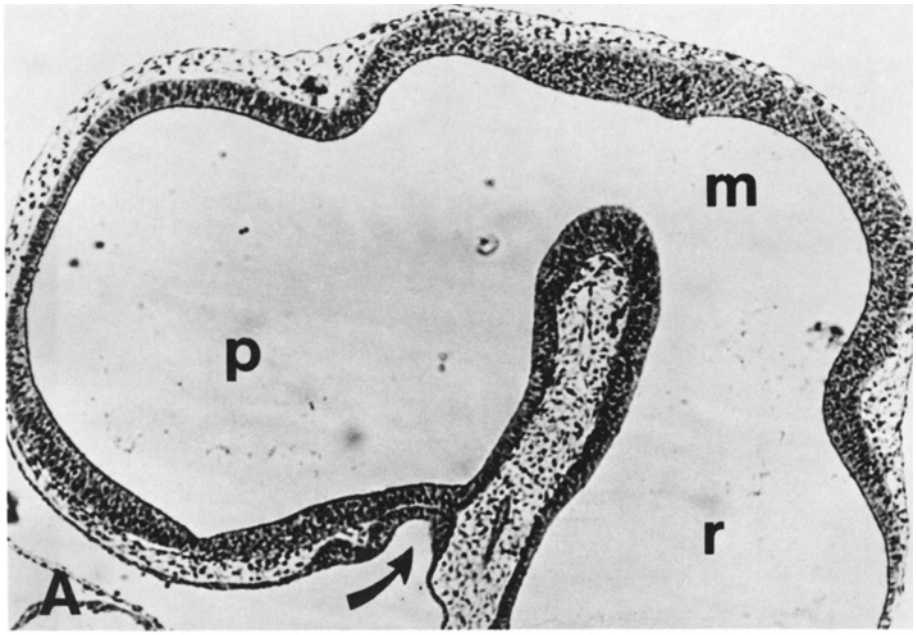


Fig. 4 A–C. Sagittal sections of rat embryos (day 11.5 of gestation) X 12 **A** Untreated control. **B** Treated twice on day 10 with 100 mg/kg acyclovir. **C** Treated once on day 10 with 200 mg/kg acyclovir



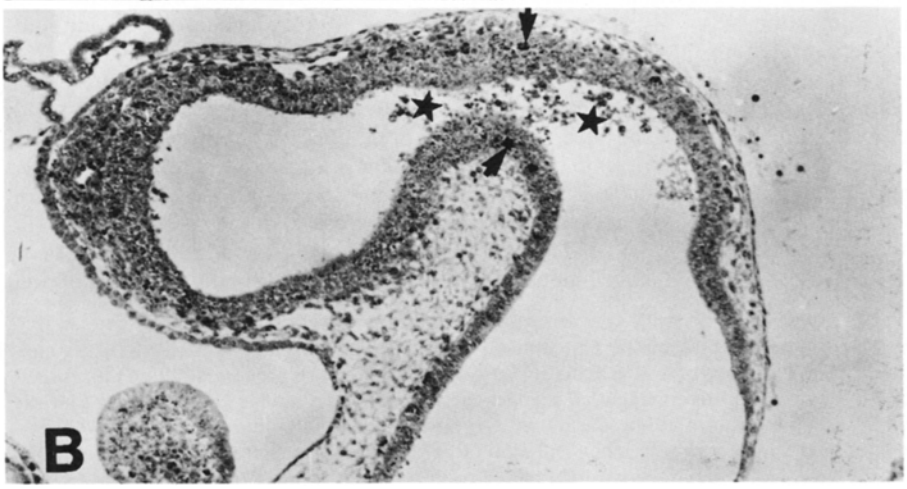


Fig. 5 A, B. Sagittal sections of rat embryos (day 11.5 of gestation) X 62. **A** Untreated control showing prosencephalon (*p*), mesencephalon (*m*), rhombencephalon (*r*) and Rathke's pouch (↑). **B** Treated once on day 10 with 200 mg/kg acyclovir. Distinct decrease of skull and brain anlage. Numerous necroses (*) in the neuroepithelium and prospective ventricular system (*)

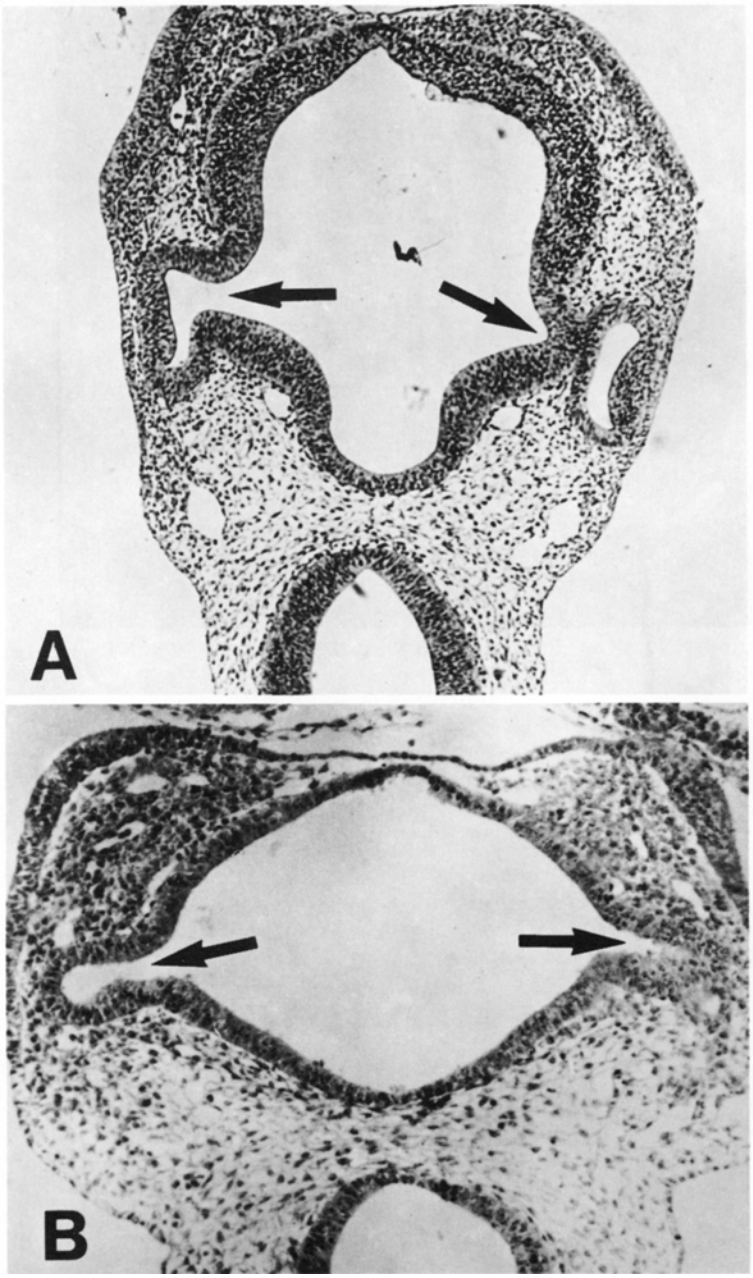


Fig. 6A, B. Cross-section of the head of a rat embryo (day 11.5 of gestation) in the region of the eye processes (↑). X 72. **A** Untreated control. Cup-shaped eye processes. **B** Treated once on day 10 with 200 mg/kg acyclovir. No formation of eye-cup, thinner neuroepithelium, flattening of the diencephalon anlage

clovir in the rat and other species. After s.c. injection of a single dose of 25 mg/kg 95% of the drug was excreted unchanged in the urine. The pharmacokinetics of acyclovir in the rat after i.v. application (25 mg/kg) could be defined by a three-compartment open model with a rapid distribution phase. The 14C half-life in the alpha phase was 1.2 h. The amount of radioactivity in the plasma 30 min after injection corresponded to 41 μM; after 12 h the level was equivalent to 0.2 μM acyclovir. Beyond this time the terminal half-life of the drug was extended to 14.4 h.

The HPLC analysis of the plasma samples in our studies shows that for a given dose level the plasma concentrations do not correlate with the embryotoxic effect. As seen from the pharmacokinetic studies by Bochert et al. (1987), the mean maximum peak concentrations after s.c. injection of 100 mg/kg or 200 mg/kg body wt can be measured after 90 min; however, the individual peak concentration was measurable between 45 and 90 min (unpublished data). A single sample at 60 min after administration gives insufficient information about the kinetics in an individual

Fig. 7A–D. Rat embryos of day 11.5 after single treatment with 200 mg/kg acyclovir on day 10. **A** Telencephalon vesicles, missing mesenchyme (*) between outer ectoderm (↓) and neuroepithelium (*E*). X 160. **B** Lateral sagittal section with eye processes (*), no cup formation yet, cell debris (↓) in the neuroepithelium and surrounding mesenchyme, pharyngeal arches (*A*), heart anlage (*H*), olfactory placode (*open arrow*). X 134. **C** Eye process (*), shortened, no cup formation, numerous necroses (↓) in the neuroepithelium, prospective ventricle of the diencephalon (*D*). X 134. **D** Oblique section of the wall of the rhombencephalon (*R*) with numerous necroses, mesenchyme (*M*), no changes. X 134

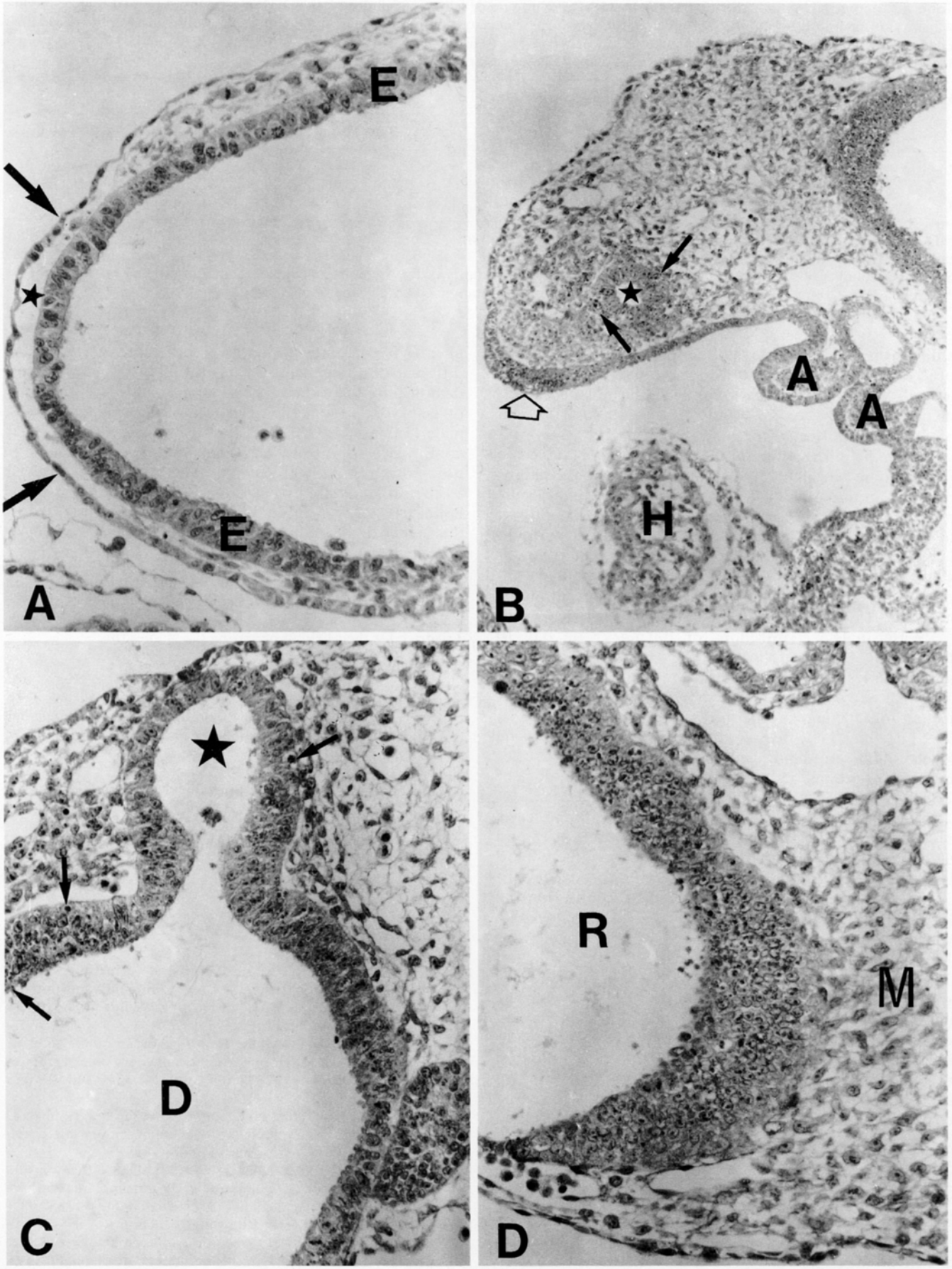

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Table 8. Comparison of the effects of acyclovir on rat embryonic development under in vivo and in vitro conditions. In vitro: median values and first and third quartile are given in the table. The concentration of acyclovir in the culture medium was 22.5 mg/l (100 μM). Data from Klug et al. (1985a). In vivo: treatment of dams on day 10 of pregnancy with three s.c. injections of 100 mg/kg body wt at 7.00, 12.00 and 17.00 hours. Evaluation of the embryos on day 11.5 of gestation

Experimental conditions		Evaluation of embryos (day 11.5)					
		n	*CR*	Somites	Protein	Score	Abnormal (%)
In vitro	Control*	44	3.36 (3.18/3.60)	26 (25/27)	235 (176/284)	37 (35/38)	0
	22.5 mg/l*	19	3.00 (2.82/3.36)	26 (25/27)	138 (122/175)	32 (31/33)	95
In vivo	3 × 100 mg/kg**	31	3.12 (2.88/3.18)	24 (20/25)	140 (86/166)	34 (32/35)	100

* Data by Klug et al. (1985a); concentration of acyclovir in the culture medium: 22.5 mg/l (= 100 μM); median values are given in this table; the figures in brackets represent the first and third quartile; *n* = number of embryos evaluated; *CR* = crown-rump length (mm); Somites = number of somite pairs; Protein = protein content (μg/embryo); for "Score" and culture conditions cf. Klug et al. (1985b)
** Three s.c. injections of acyclovir to pregnant rats on day 10 of gestation 7.00, 12.00 and 17.00 h

animal: the drug level may still be rising, it may be at the peak or it may already be decreasing. A closer "drug monitoring" would be necessary although during the experiments described here, we avoided this to limit the amount of "experimental stress" done to the animals.

From the information on the plasma levels obtained so far, it can be postulated that maternal peak concentrations must be around 100 mg/l (444 μM) to obtain teratogenic effects in rat fetuses. Eight injections of 50 mg/kg body wt on days 9, 10 and 11 of gestation reduce the crown-rump length of the fetuses but are not sufficient to induce teratogenic effects which are detectable with the methods applied in the studies described here. The concentrations measured 1 h after this dose range from 19.1 to 40.0 mg/l.

With single s.c. injections of 100 mg/kg on day 10 leading to 1 h post-application levels up to 80.1 mg/l, no abnormalities were detectable in the fetuses on day 11.5. The pharmacokinetic analysis shows that after a single injection of 100 mg/kg the mean peak concentration is 99.2 ± 21.7 mg/l (Bochert et al. 1987). Mean maternal drug levels which are significantly higher are obtained after multiple injections of 100 mg/kg or with a single dose of 200 mg/kg.

Tucker and Clive (1983) measured mean plasma levels of acyclovir in three rats on day 15 of gestation. At 15 and 60 min after s.c. injection of 25 mg/kg body wt they found levels of 16 ± 3 μg/ml and 9 ± 0.6 μg/ml, respectively.

At the same time intervals after drug application the levels in the amniotic fluid and in the fetal homogenate were determined: amniotic fluid: 0.7 ± 0.1 μg/ml and 1.0 ± 0.2 μg/ml; fetal homogenate: 2.0 ± 0.04 and 5.0 ± 1.1 μg/ml. Similar data have been published elsewhere by the same working group (Moore et al. 1983). The authors tested lower doses as well and found "increasing concentrations in fetal homogenate in direct proportion to increasing doses given to the dams". With the assumption that this occurs proportionally at higher doses as well, a fetal concentration of approximately 20 μg/g wet wt can be estimated after injection of 100 mg/kg body wt. With two or three injections of this dose we found similar effects to Klug et al. (1985a), with concentrations between 11.3 and 22.5 μg acyclovir/ml culture medium. This underlines the value of the whole-embryo culture system for the predictability of the teratogenic potential of acyclovir.

To our knowledge, this is the first example of an effect which, after being observed in vitro, has been predicted to also have the potential to occur in vivo and has subsequently been demonstrated to be inducible in vivo. With different dose regiments on day 10 of gestation we could demonstrate that exactly the same morphological outcome of embryos is inducible in experiments using intact animals (Table 8, Fig. 2). This may be considered a mutual confirmation of the in vitro and in vivo investigations. It is now proven that the effects observed in vitro are induced by the drug and are by no means "artifacts". On the other hand, it seems very probable that the effects described in this paper are specifically drug-related and not a consequence of maternal toxicity.

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References

Andersson J, Britton S, Ernberg I, Andersson U, Henle W, Sköldenberg B, Tisell A (1986) Effect of acyclovir on infectious mononucleosis: A double-blind, placebo-controlled study. J Infect Dis 153: 283–290

Bochert G, Rahm U, Schwabe R, Meister R (1987) Plasma concentrations of acyclovir in pregnant rats. Naunyn-Schmiedeberg's Arch Pharmacol 335 (Suppl): R29 (Abstr. No. 116)

De Miranda P, Krasny HC, Page DA, Elion GB (1981) The disposition of acyclovir in different species. J Pharmacol Exp Ther 219: 309–315

Elion GB (1982) Mechanism of action and selectivity of acyclovir. Am J Med 73 (Suppl): 7

Furuhama K, Onodera T (1983) A simple technique for repeated blood collection from the tail vein of the rat. Toxicol Sci 8: 161–163

Hayden FG, Douglas RG Jr (1985) Antiviral agents. In: Mandell GL, Douglas RG Jr, Bennett JE (eds) Principles and practice of infectious diseases, 2nd Edition, John Wiley & Sons, New York Chichester Brisbane, pp 270–286

Heinrich-Hirsch B, Jacob-Müller U (1987) Studies on the embryotoxic effect of acyclovir on the development of chick embryos. Naunyn-Schmiedeberg's Arch Pharmacol 335 (Suppl): R29 (Abstr. No. 115)

Klug S, Lewandowski C, Blankenburg G, Merker H-J, Neubert D (1985a) Effect of acyclovir on mammalian embryonic development in culture. Arch Toxicol 58: 89–96

Klug S, Lewandowski C, Neubert D (1985b) Modification and standardization of the culture of early postimplantation embryos for toxicological studies. Arch Toxicol 58: 84–88

Moore HL Jr, Szczech GM, Rodwell DE, Kapp RW Jr, De Miranda P, Tucker WE Jr (1983) Preclinical toxicology studies with acyclovir: teratologic, reproductive and neonatal tests. Fund Appl Toxicol 3: 560–568

Neubert D, Blankenburg G, Chahoud I, Franz G, Herken R, Kastner M, Klug S, Kröger J, Krowke R, Lewandowski C, Merker H-J, Schulz T, Stahlmann R (1986) Results of in vivo and in vitro studies for assessing prenatal toxicity. Environ Health Perspectives 70: 89–103

Shepp DH, Dandliker PS, Meyers JD (1986) Treatment of varicella-zoster virus infection in severely immunocompromised patients. N Engl J Med 314: 208–212

Smith RL, Walker DD (1985) High-performance liquid chromatographic determination of acyclovir in serum. J Chromatogr 343: 203–207

Stahlmann R, Chahoud I (1987) Teratogenic potential of acyclovir in rats. Naunyn-Schmiedeberg's Arch Pharmacol 335 (Suppl): R 29 (Abstr. No. 114)

Tucker WE Jr, Clive D (1983) Preclinical toxicology profile of acyclovir: an overview. Poster representation at 2nd International Acyclovir (Zovirax) Symposium, London 1983

Tucker WE Jr, Macklin AW, Szot RJ, Johnston RE, Elion GB, De Miranda P, Szczech GM (1983) Preclinical toxicology studies with acyclovir: Acute and subchronic tests. Fund Appl Toxicol 3: 573–578

Whitley RJ, Alford CA, Hirsch MS, Schooley RT, Luby JP, Aoki FY, Hanley D, Nahmias AJ, Soong S-J (1986) NIAID Collaborative Antiviral Study Group, 1986, Vidarabine versus acyclovir therapy in herpes simplex encephalitis. N Engl J Med 314: 144–149

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