Eur J Cancer, Vol. 29A, No. 1, pp. 96–101, 1993.
Printed in Great Britain

0964–1947/93 $5.00 + 0.00

In vitro and *in vivo* Antitumoral Activity of Alkylphosphonates

Uwe J. Ries, Eduard A.M. Fleer, Anne Breiser, Clemens Unger, Juri Stekar, Karin Fenneberg and Hansjoerg Eibl

Hexadecylphosphocholine is a new antitumour agent with a highly selective activity in chemically induced mammary tumours. It was suggested, that hexadecylphosphocholine is a pro-drug, cleavable by phospholipases C and/or D, creating hexadecanol or hexadecylphosphate as the active principle. To test this hypothesis, the antineoplastic activity of three alkylphosphonates, cleavable either by phospholipase C or D, are compared with those of the parent compound, hexadecylphosphocholine. Cell culture experiments, in which radiolabelled alkylphosphonates were incubated with a neoplastic cell line, showed no metabolism even after 3 days of incubation. In *in vivo* experiments with dimethylbenzanthracene-induced rat mammary carcinomas, all three alkylphosphonates showed antineoplastic activity, although none of them reached the high activity of hexadecylphosphocholine. These results indicate that the antitumoral activity of alkylphosphocholines and alkyl lysophosphatidylcholines is due to direct toxicity and not dependent on metabolism by phospholipases C or D or related enzymes.
Eur J Cancer, Vol. 29A, No. 1, pp. 96–101, 1993.

INTRODUCTION

ALKYLPHOSPHOCHOLINES (ALP) REPRESENT a new class of antitumour ether lipid analogues with remarkable activity *in vitro* and *in vivo* [1–3]. The different biological effects described for antineoplastic etherlipids include direct cytotoxicity [1, 4], induction of cell differentiation [5, 6], inhibition of tumour growth and invasion [7], damage of cell membranes [8, 9] and activation of macrophages [10]. However, the precise mode of antitumoral action is not yet understood. The biological effects of hexadecylphosphocholine (**1**) are most intensively studied. This compound is strictly not a (ether)-lysophospholipid since it lacks the glycerol backbone. Most probably, it contains the essential structural elements for antineoplastic activity [11]. Using lymphomic Raji cells in metabolic studies with **1**, ^{3}H-labelled in the methyl groups of choline, a transfer of the phosphocholine group from hexadecylphosphocholine to diacylglycerol was observed [1], leading to the formation of (diacyl)phosphatidylcholine. The amount of radiolabel transferred to diacylglycerol seemed to correlate with the percentage of Trypan blue-positive cells, and therefore, a possible relation between phosphocholine transfer and cell death was postulated. To verify this hypothesis we have synthesised alkylphosphonates, which are only cleavable by either phospholipase C or phospholipase D (Fig. 1).

These compounds differ from **1** by phosphorus–carbon bonds either between the alkyl chain and the phosphorus [cholinylhexadecylphosphate (**2**)] or between the phosphorus and the quarternary ammonio group [hexadecyl 2-(*N*,*N*,*N*-trimethylammonio)ethylphosphonate (**3**) and hexadecyl 3-(*N*,*N*,*N*-trimethylammonio)propylphosphonate (**4**)]. Compounds **3** and **4** differ only in the phosphorus–nitrogen distance, as a result of which an increased polarity of **3** compared to **4** and **1** was observed [12].

In the present report we describe the substrate properties of alkylphosphonates for phospholipases C and D, the uptake and metabolism of these compounds in lymphomic Raji and leukaemic U 937 cells and their cytotoxic activity *in vitro* as well as their antineoplastic activity *in vivo*. Although we used phospholipase C from *Bacillus cereus* and phospholipase D from cabbage to test the susceptibility of the compounds for these enzymes, cellular enzymes should show similar effects since the compounds are synthesised such that they are only degraded by either phospholipase C or phospholipase D. For instance, compound **2** misses the oxygen atom between the long aliphatic chain and phosphorus which is necessary for hydrolysis by phospholipases C, whereas compounds **3** and **4** are missing the oxygen atom between phosphorus and the quaternary ammonium base, necessary for hydrolysis by phospholipases D. Therefore, we believe that these investigations allow us to estimate the importance of phospholipase C and D or related enzymes, for antitumoral activity.

Correspondence to E.A.M. Fleer.
U. J. Ries is at the Department of Chemical Research, Dr. Karl Thomae GmbH, Birkendorfer Strasse 65, W-7950 Biberach/Riss; E.A.M. Fleer, A. Breiser and C. Unger are at the University Clinics of Goettingen, Department of Internal Medicin, Division of Haematology/Oncology, Robert-Koch-Strasse 40, W-3400 Goettingen; J. Stekar and K. Fenneberg are at the Department of Cancer Research and Development, ASTA Pharma AG, Weissmuellerstrasse 45, W-6000 Frankfurt 1; and H. Eibl is at the Max-Planck-Institute for Biophysical Chemistry, Department 145, Membrane Biophysics, Am Fassberg, W-3400 Goettingen, F.R.G.
Revised 9 Apr. 1992; accepted 29 June 1992.

MATERIALS AND METHODS

Materials

Compounds **1**, **2**, **3** and **4**, as well as the radiolabelled compounds **1**, **2** and **3** were prepared as described elsewhere [12]. Other chemicals were of analytical grade and were used without further purification. Phospholipase C from *Bacillus cereus* (2000 U/mg) was from Boehringer Mannheim (FRG). Bovine serum albumin (BSA) was from Sigma (Munich, FRG).

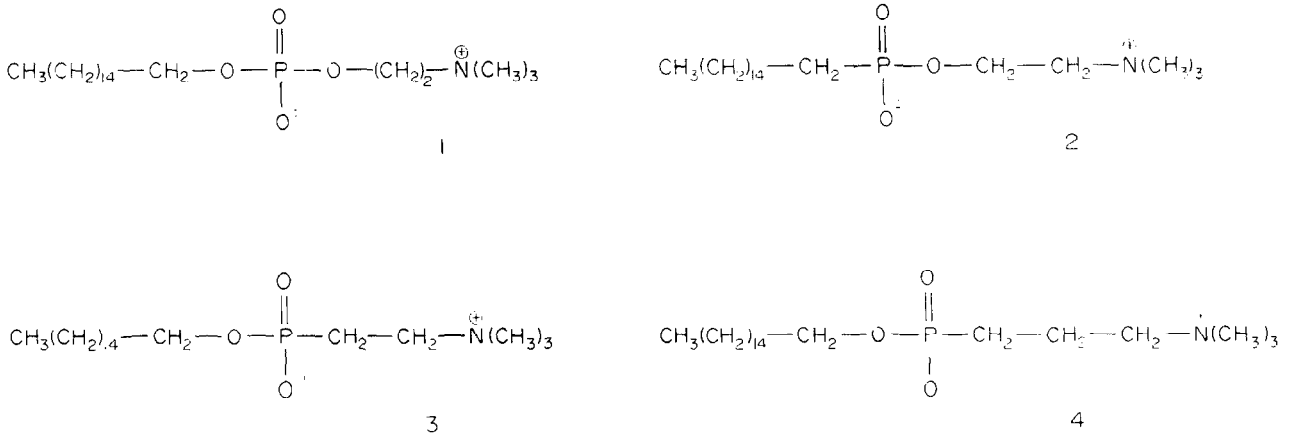

Fig. 1. Chemical structures of hexadecylphosphocholine (1), cholinyl-hexadecylphosphonate (2) (missing the oxygen atom between the aliphatic chain and phosphorus, necessary for phospholipase C degradation), hexadecyl-(2-trimethylamino)ethylphosphonate (3) and hexadecyl-(3-trimethylamino)propylphosphonate (4) (both missing the oxygen atom between phosphorus and the quaternary ammonium base, necessary for phospholipase D activity).

Methods

Phospholipase C and phospholipase D assays. Incubations with phospholipase C from *Bacillus cereus* were carried out as described elsewhere [13]. Phospholipase D was isolated from white cabbage according to Kovatchev and Eibl [14]. The enzymic activity was determined by re-esterification in the presence of methanol [14]. Methyl phosphates or methyl phosphonates, respectively, which were formed during the enzymic reaction, were isolated by preparative thin layer chromatography (TLC). The bands containing the reaction products were extracted from silica gel and the amount of lipid phosphorus was then measured according to Eibl and Lands [15]. Conditions were chosen such that no more than 10% of substrate was degraded. Under these conditions enzyme activity was linear with respect to enzyme concentration and time.

Cell cultures. Raji-cells (B-lymphoblastic lymphoma) and U 937-cells (promyelocytic leukemia) were grown in RPMI 1640 medium (GIBCO, Glasgow, UK), supplemented with 10% fetal calf serum, streptomycin (128 mg/l) and penicillin (10^5 U/l), at 37°C under an atmosphere of 5% CO_2 in humidified air.

Determination of cell viability and proliferation. Incubations of cells (10 ml, 2×10^5 cells/ml) were performed with alkylphosphonates and hexadecylphosphocholine using concentrations between 2.5 μg/ml (ca. 6 μmol/l) and 40 μg/ml (ca. 94 μmol/l). After the indicated time periods, aliquots of the cell suspension were counted in a Neubauer cell-counting chamber and tested for viability using the Trypan blue dye exclusion test [16]. The proliferative capacity of the cells was also determined by [^{3}H]thymidine uptake. Three-fold to five-fold replicates of 10^4 tumour cells/well were incubated with the compounds at concentrations between 1 and 100 μmol/l for 48 h at 37°C in 96-well microculture trays (Greiner, Nürtingen, FRG). A 6-h pulse with [^{3}H]thymidine at 18.5 KBq/well (specific activity 33.3 TBq/mmol, Amersham Buchler, Braunschweig, FRG) was performed at the end of the culture period. Cells were collected with a cell harvester (PDH, Cambridge Technology Inc., Cambridge, Massachusetts) and filter dishes were air dried. Radioactivity was measured by liquid scintillation counting.

Uptake and metabolism in Raji-cells. Incubations of Raji-cells with radiolabelled **1, 2** and **3** were performed at a concentration of 10 μg/ml (ca. 24 μmol/l). At indicated time points cells were counted and viability control was performed as written above. Then the cell suspension was diluted with 10 ml bovine serum albumin (BSA) solution (0.05% in 0.9% NaCl) and centrifuged at 1000 *g* for 10 min. The supernatant was collected, and the cell pellet was washed twice again with 10 ml BSA solution. Subsequently, the cell pellet was extracted twice according to Bligh and Dyer [17]. The extracts were combined; 2.5 ml water and 2.5 ml chloroform were added for phase separation. The chloroform layer (lower phase) was collected, and the water phase was re-extracted with 5 ml chloroform. Aliquots of the water and chloroform phases were taken for radioactivity counting. The sum of the radioactivity in both phases was used to calculate the amount of alkylphosphonate or hexadecylphosphocholine, respectively, taken up by the cells. The chloroform phases were dried under a stream of nitrogen and redissolved in 200 μl of chloroform/methanol (9:1, v/v).

The organic material was then applied to a thin layer chromatography plate, and the plate was developed in chloroform/methanol/aqueous ammonia (25%) (60/40/4, by volume). After drying, the plate was scanned for radioactive products in an Automatic Linear Analyzer LB 284 complemented with Data System Chroma (Berthold, Wildbad, FRG).

Antitumour activity in vivo. The *in vivo* antineoplastic activity of alkylphosphonates was determined on dimethylbenzanthracene (DMBA)-induced rat mammary carcinomas [5, 18] in comparison to **1**. Daily oral treatment for 14 days with alkylphosphonates or **1** were started when DMBA-induced mammary carcinomas of adult female Sprague–Dawley rats attained a mass of 0.8–1.4 g.

RESULTS

Substrate properties for phospholipases C and D

The structures of hexadecylphosphocholine and the three phosphonate derivatives (**2, 3** and **4**) are shown in Fig. 1. The three alkylphosphonates show the expected substrate behaviour against phospholipase C and phospholipase D. Compound **2**, missing the oxygen atom necessary for hydrolysis by phospholipase C, cannot be degraded by this enzyme. Compounds **3** and **4**, with the oxygen atom between phosphorus and the quaternary ammonium base missing, cannot be hydrolysed by phospholipases D. As shown in Table 1, compound **2** is only degradable by phospholipase D, but not by phospholipase C.

Table 1. Substrate properties of hexadecylphosphocholine (**1**), *its alkylphosphonate derivatives* (**2–4**), 1-O-*hexadecyl-2-acetyl-sn-G-3-PC (PAF) and* 1,2-*dipalmitoyl-sn-G-3-PC for phospholipases C and D*

Substrate	Phospholipase C (*Bacillus cereus*) (nmol/mg.min)	Phospholipase D (white cabbage) (nmol/mg.min)
1,2-Dipalmitoyl-sn-G-3-PC	149.950	14.7
1-*O*-hexadecyl-2-acetyl-sn-G-3-PC (PAF)	1.270.000	4.0
1	38	9.8
2	0	5.8
3	36	0
4	25	0

The activity of phospholipase D for **2** is in the same order of magnitude as for compound **1**, 1-*O*-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine (PAF) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (lecithin). In contrast, alkylphosphonates **3** and **4** are not degraded at all by phospholipase D. The latter two phosphonates are degradable by phospholipase C, but, as **1**, are poor substrates compared with PAF, which is an excellent substrate for phospholipase C [12]. These results are a strong indication, that alkylphosphonates **2**, **3** and **4** will be metabolised in a defined way, although especially phospholipase C seems to be sensitive to changes in the molecular structure.

Cytotoxic activity in vitro

Both the inhibition of cell proliferation and the cytotoxic effects of the different alkylphosphonates on human lymphomic Raji and leukemic U 937 cells in cell cultures were determined. Cell viability was measured using the Trypan blue dye exclusion assay [16].

The antiproliferative activity was determined by two different methods: by cell counting as well as by the loss of [^{3}H]thymidine incorporation in the cellular DNA. Both the 50% growth inhibition concentration (IC_{50}) in the cell counting as well as in the [^{3}H]thymidine assay and the half lethal concentration (LC_{50}) in the Trypan blue dye exclusion assay were interpolated after 48 h of cell incubation, when cell growth of control incubations was in a logarithmic phase. Figure 2 shows the antiproliferative activity of the alkylphosphonates in comparison to compound **1** against Raji cells. Compounds **1** and **3** show nearly identical activities, whereas **2** and **4** are slightly less active, particularly at low phosphonate concentrations. In Fig. 3 the effects of all four compounds on cell viability are shown. All substances show a distinct cytotoxicity, but compound **2**, which is not degradable by phospholipase C, seems to be less toxic against Raji-cells. The values of IC_{50} and LC_{50} resulting from cell counting experiments as in Figs 2 and 3 and the IC_{50} value obtained from the [^{3}H]thymidine assays are shown in Table 2. All values are taken after 48 h of drug exposure and show that the three alkylphosphonates, like **1**, have growth inhibiting activities. In the thymidine assay, as well as in the Trypan blue dye exclusion test, **2** seems to have a weaker antineoplastic activity. Compounds **3** and **4**, which are both degradable by phospholipase C, show different effects on Raji cells. Obviously, small changes in the phosphorus–nitrogen distance of alkylphosphonates do not influence the cytotoxic activity *in vitro*, but changes in cell proliferation are clearly visible.

Most of the results described above are confirmed by incubations of U 937 cells with alkylphosphonates. These cells are shown to be highly sensitive against ALP and APC [6]. As shown in Table 3, compounds **1** and **3** are highly active against U 937 cells, whereas compound **2** is less active again by a factor of two in the IC_{50} and LC_{50} values.

Uptake kinetics in Raji cells

Raji cells were incubated with radiolabelled **1**, **2** and **3** in a concentration of 10 μg/ml (ca. 24 μmol/l). The amounts of compound taken up were calculated from the determined amounts of radioactivity in the cells and the specific activity of the incubated compounds, and related to 10^6 cells. The results are shown in Fig. 4, where these values are plotted against time. The uptake kinetics of the tested compounds show a distinct difference. In 24 h Raji cells take up twice the amount of **1** and **3** compared with **2**. After 48 h, **1** and **3** show a saturation of uptake. Compound **2** is taken up at a slower rate, but after 72 h the cells contain the same amounts of **2** and **3**. The difference in uptake rates might explain the different cytotoxicities observed for **1**, **2** and **3**.

Because of its delayed uptake, compound **2** exhibits cytotoxic activity at a later point in time as compounds **1** and **3**. It has to be noted, however, that even after 72 h of incubation, the

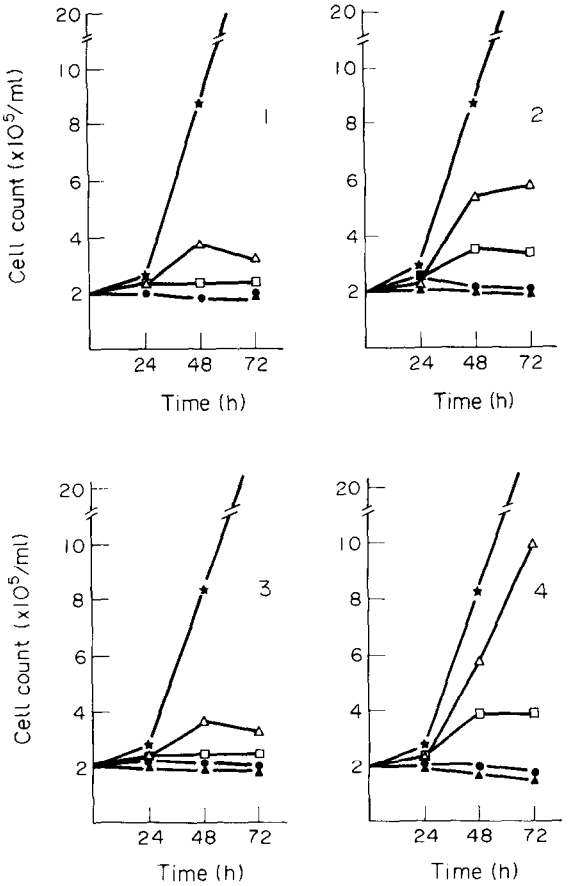

Fig. 2. Effects of compounds 1–4 on the cell growth of Raji cells. 2 × 10^5 cells/ml were grown in RPMI medium in the presence of 5–30 μg/ml of compounds 1, 3 and 4 and 5–40 μg/ml of compound 2. After indicated time periods cells were counted in a Neubauer cell counting chamber. All data are mean values from three to five single experiments. Stars: control cells (no addition); open triangles: 5 μg/ml; open squares: 10 μg/ml; closed circles: 20 μg/ml; closed triangles: 30 μg/ml (1, 3, 4) or 40 μg/ml (2). Standard errors are 10%.

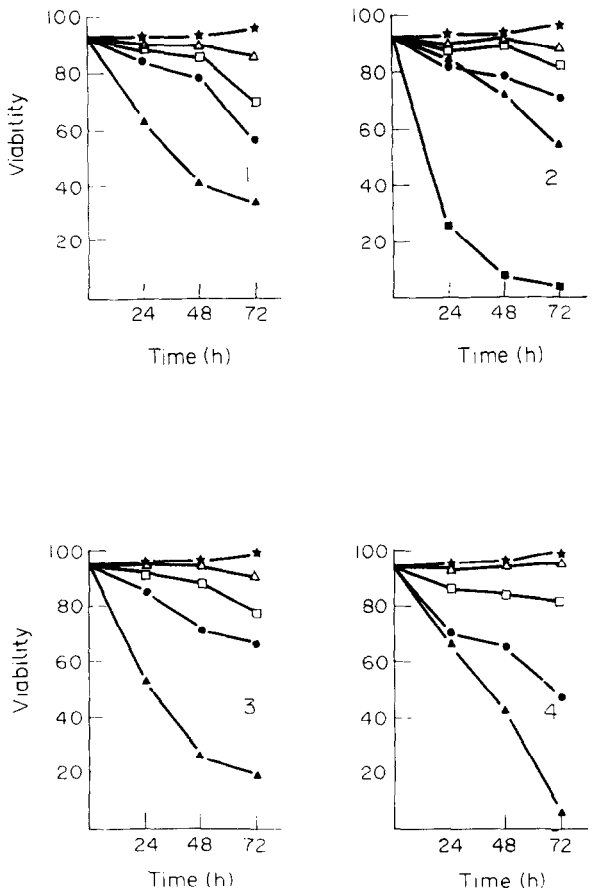

Fig. 3. Effects of compounds 1–4 on the viability (%) of Raji cells. 2 × 10^5 cells/ml were grown in RPMI medium in the presence of 5–30 μg/ml of compounds 1, 3 and 4 and 5–40 μg/ml of compound 2. After indicated time periods cells were counted in a Neubauer cell counting chamber. All data are mean values from three to five single experiments. Stars: control cells (no addition); open triangles: 5 μg/ml; open squares: 10 μg/ml; closed circles: 20 μg/ml; closed triangles: 30 μg/ml (1, 3, 4) or 40 μg/ml (2). Standard errors are 10%.

Table 2. Antiproliferative and cytotoxic activity of hexadecylphosphocholine (1) and alkylphosphonates (2–4) against Raji cells after 48 h of incubation

Substance	IC_{50}* Thymidine (μg/ml)	IC_{50}† Cell count (μg/ml)	LD_{50}‡ Trypan blue (μg/ml)
1	6.6 (3.0)	3.0 (1.0)	28 (3)
2	9.2 (2.9)	5.0 (1.0)	35 (4)
3	5.4 (2.4)	3.0 (1.0)	25 (4)
4	not determined	9.0 (2.0)	28 (3)

Values are expressed as mean (S.D.).
* Obtained by [^{3}H]thymidine uptake experiments.
† Obtained by cell counting.
‡ Obtained by Trypan blue exclusion test.
The data for [^{3}H]thymidine uptake are mean values from nine single experiments, all other data are mean values from three to five single experiments.

Table 3. Antiproliferative and cytotoxic activity of hexadecylphosphocholine (1) and alkylphosphonates (2 and 3) against U 937 cells after 48 h of incubation

Substance	IC_{50}* Thymidine (μg/ml)	IC_{50}† Cell count (μg/ml)	LC_{50}‡ Trypan blue (μg/ml)
1	2.8 ± 0.3	2.5 ± 0.5	7.5 ± 1.0
2	5.1 ± 1.1	4.0 ± 1.0	15.0 ± 2.0
3	2.6 ± 0.5	2.5 ± 0.5	6.0 ± 1.0

Values are expressed as mean (S.D.).
* Obtained by [^{3}H]thymidine uptake experiments.
† Obtained by cell counting.
‡ Obtained by Trypan blue exclusion test.
The data for [^{3}H]thymidine uptake are mean values from five single experiments, all other data are mean values from three single experiments.

cytotoxic effects of 2 are less pronounced than these for compounds 1 and 3, although at 72 h compounds 2 and 3 are incorporated to a higher amount in Raji cells than compound 1. It should be mentioned that the critical micellar concentration (cmc) of the compounds 1 to 4 differ only slightly with values between 8 and 10 μmol/l [12, 19]. These small differences in cmc values cannot explain the differences in uptake kinetics of the compounds or differences in their cytotoxicity.

Metabolism in Raji cells

The metabolic studies of 2 and 3 in Raji cells were performed in parallel to the uptake experiments described above. After the indicated time periods the cells were centrifuged and washed with 0.05% BSA solution to remove unincorporated phosphonates. After Bligh and Dyer extraction, the radioactivity in the aqueous phase as well as in the organic phase was measured. In all experiments about 95% of radioactivity was found in the

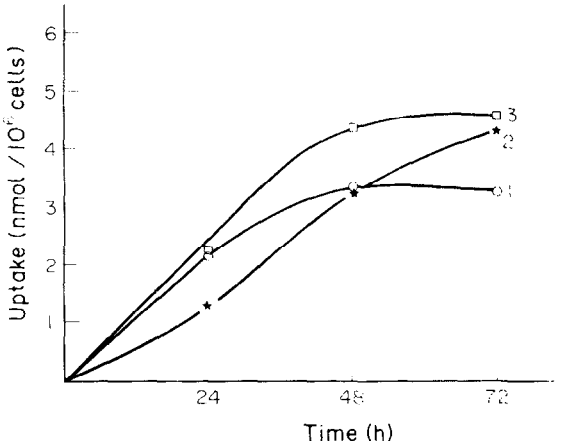

Fig. 4. Uptake kinetics of radiolabelled compounds 1, 2 and 3 in Raji cells. Cells were incubated in RPMI-medium containing 10 μg/ml of radiolabelled compounds. At indicated time intervals, the cells were harvested directly after dilution of the cell suspension with 0.05% BSA-solution in 0.9% NaCl. After being washed twice with BSA, the cell pellet was extracted according to Bligh and Dyer (1969), and the organic phase was counted for radioactivity. All data are mean values from at least three duplicate experiments. Open circles: compound 1; stars: compound 2 and squares: compound 3. Standard errors are 10%.

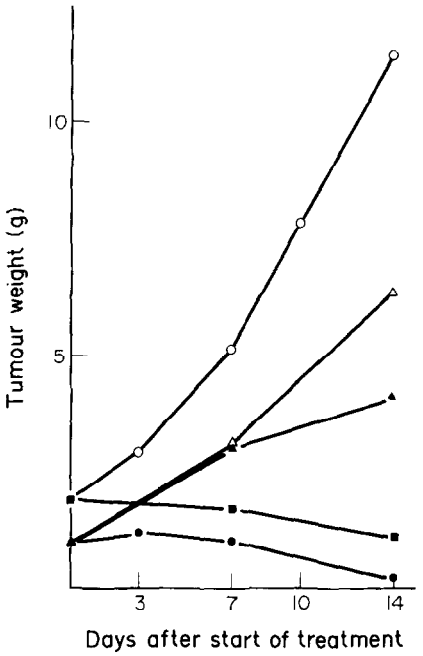

Fig. 5. Antineoplastic activity *in vivo* of compounds 1, 2, 3 and 4. The antitumoral activity was determined on rats bearing chemically induced mammary carcinomas. Induction of mammary carcinomas is described in Materials and Methods and by Hilgard *et al.* [3, 5]. After the tumours reached a size of between 0.8 and 1.4 g, treatment was started with: open circles: no additions (control); closed circles: compound 1, 5 times weekly at 32 mg/kg body weight; squares: compound 2, 4 times 100 mg/kg body weight; closed triangles: compound 3, 4 times 68 mg/kg body weight; and open triangles: compound 4, 4 times 100 mg/kg body weight. Tumour growth was assessed after 3, 7, 10 or 14 days by estimating the weight of the mammary carcinoma. Standard errors are 15%.

organic phase. The total amount of radioactivity in the water phase was about 0.037 KBq, at most. Because of this very low activity no substance could be identified by TLC. The found activity most probably corresponds to the small amount of original compounds that partitions in the water phase. Bligh and Dyer extractions with **1** in the absence of cell material shows a similar distribution between chloroform and water phases (data not shown; about 3% in the water phase).

In contrast to metabolic studies with compound **1**, the Bligh and Dyer extracts did not show any metabolic products of the radioactive compounds **2** and **3**, and in all TLC runs only the parent compounds could be identified as radioactivity carriers, indicating less than 1% degradation. For compound **1**, 10–15% of the radiolabel was recovered as (diacyl)phosphatidylcholine, indicating that metabolism does take place in Raji cells. Since the alkylphosphonates are not degraded, and since the alkylphosphonates show cytotoxic activity more or less comparable to hexadecylphosphocholine, which is degraded, it is clear that cytotoxicity in *in vitro* experiments is not necessarily related to metabolism of the alkylphosphonates and, most likely, not a prerequisite for cytotoxicity of alkyl lysophosphatidylcholines and alkylphosphocholines.

Antineoplastic activity in vivo

The *in vivo* antitumoral activities of compounds **2**, **3** and **4** on rats bearing chemically induced mammary carcinomas were compared with the antineoplastic activity of compound **1** in the same tumour model. Compared with control animals that were not treated with alkylphosphonates or hexadecylphosphocholine, all compounds showed distinct antitumoral activity (Fig. 5). However, the effect of the individual agents differed. Whereas compounds **3** and **4** only showed growth retardation of the mammary carcinomas, application of compound **2** for 14 days led to a regression to below the size registered at the start of therapy. Compound **1** was still more effective as compound **2**. It must be noted that compounds **2** and **4** were applied four times at a maximal tolerable dose of 100 mg/kg body weight, whereas compound **3** was administered four times at a maximal tolerable dose of 68 mg/kg body weight. Compound **1** was given 5 times weekly at a dose of 32 mg/kg body weight, a dose that is tolerated over at least 4 weeks of application. From earlier experiments it is known that a dose of four times 68 mg/kg body weight of compound **1** yields a regression of tumour mass from about 5 g at the start of application to less than 1 g after 14 days of treatment [3, 4]. We, therefore, conclude that compounds **2**, **3** and **4** show antineoplastic activity, but these compounds are less effective than compound **1**.

DISCUSSION

The properties of different alkylphosphonates in comparison with hexadecylphosphocholine were studied in different systems. It was demonstrated that compound **2** only is metabolised by phospholipase D, whereas compounds **3** and **4** are substrates for phospholipase C. Therefore, if processing by phospholipase C is an essential requirement for the antitumoral activity, compound **2** should be inactive. However, this is not observed in cell culture and in *in vivo* experiments.

Differences in the cytotoxicity of compounds **2** and **3** can be explained by differences in the uptake of these compounds in Raji cells, as can be shown by a comparison of IC_{50} and LC_{50} values with uptake kinetics. Compound **2**, a substrate for phospholipase D, is less toxic than compound **3**, a substrate for phospholipase C, probably due to the fact that after 48 h of incubation only 3 nmol of **2** but 4 nmol of **3** are incorporated into the same number of Raji cells. A similar result could be observed when U937 cells are incubated with the compounds **1**, **2** or **3**. Compound **1** is most toxic and nearly equalled by compound **3**, whereas compound **2** is less toxic. Since no metabolism is observed at all, and the cells are affected by all three compounds, a toxification of these compounds via a metabolic reaction can be excluded for these cells. This is a strong indication that the compounds themselves and not their metabolites are the active cytotoxic components. In addition, the *in vivo* experiment with tumour-bearing rats did not show any correlation with susceptibility for phospholipase C or phospholipase D hydrolysis. In these experiments, it was shown that all three alkylphosphonates have distinct antineoplastic activity, although none of the phosphonates was as potent as **1** in this tumour model.

In contrast to the *in vitro* experiments, compound **2** was more active than the other phosphonates. It is possible that compounds **3** and **4** are more susceptible to degrading enzymes in the digestive system in the rat than compound **2**. This would lead to a higher concentration of compound **2** in circulating systems as compared with the other compounds. However, all three substances were less active as antineoplastic agent than compound **1**, the parent compound. Since compounds **1**, **3** and **4** are all poor substrates for phospholipase C from *Bacillus cereus*, the degradation of these alkylphosphocholine analogues seems not to play a major role in their *in vivo* antineoplastic activity. Another explanation could be that the compounds are absorbed at different rates from the digestive tract, thereby leading to differences in the blood level.

1. Fleer EAM, Unger C, Kim D-J, Eibl H. Metabolism of ether phospholipids and analogs in neoplastic cells. *Lipids* 1987, **22**, 856–861.
2. Muschiol C, Berger MR, Schuler B, *et al.* Alkyl phosphocholines: toxicity and anticancer properties. *Lipids* 1987, **22**, 930–934.
3. Hilgard P, Stekar J, Voegeli R, *et al.* Characterization of the antitumor activity of hexadecylphosphocholine (D 18506). *Eur J Cancer Clin Oncol* 1988, **24**, 1457–1461.
4. Schick HD, Berdel WE, Fromm M, *et al.* Cytotoxic effects of etherlipids and derivatives in human nonneoplastic bone marrow cells and in leukemic cells *in vitro*. *Lipids* 1987, **22**, 904–910.
5. Hilgard P, Harlemann JH, Voegeli R, Maurer HR, Echarti D, Unger C. The antineoplastic activity of hexadecylphosphocholine (HPC) is associated with tumor cell differentiation. *Proc Am Ass Cancer Res* 1989, **30**, 2310.
6. Hochhut C, Berkovic D, Eibl H, Unger C, Doenecke D. Effects of antineoplastic phospholipids on parameters of cell differentiation in U937 cells. *J Cancer Res Clin Oncol* 1990, **116**, 459–466.
7. Storme GA, Bruyneel EA, Schallier DC, Bolscher JG, Berdel WE, Mareel MM. Effect of lipid derivatives on invasion *in vitro* and on surface glycolipids of three rodent cell types. *Lipids* 1987, 22, 847–850.
8. Noseda A, Godwin PL, Modest EJ. Effects of antineoplastic etherlipids on model and biological membranes. *Biochim Biophys Acta* 1988, **945**, 92–100.
9. Noseda A, White JC, Godwin PC, Jerome WG, Modest EJ. Membrane damage in leukemic cells induced by ether and ester lipids: An electron microscopic study. *Exp Mol Pathol* 1989, **50**, 69–83.
10. Munder PG, Weltzien HU, Modolell M. Lysolecithins: a new class of immunopotentiators. In Miescher PA, ed. *Immunopathology, VIII. Int. Symposium*. Basel, Schwabe und Co., 1976, 411–424.
11. Fleer EAM, Kim D-J, Nagel GA, Eibl H, Unger C. Cytotoxic activity of lysophosphatidylcholine analogues on human leukemic Raji cells. *Onkologie* 1990, **13**, 295–300.
12. Ries UJ, Fleer EAM, Eibl H. Synthesis of alkylphosphocholines, a new class of antineoplastic agents. *Chem Phys Lipids* 1992, **61**, 225–234.
13. Unger C. *Alkylphosphocholine und Analoga. Entwicklung einer neuen Substanzgruppe mit antineoplastischer Wirkung*. Stuttgart, Thieme, 1989, 1–106.
14. Kovatchev S, Eibl H. *Enzymes of Lipid Metabolism*. New York, Plenum Press, 1978, 221–226.
15. Eibl H, Lands WE. A new, sensitive determination of phosphate. *Analyt Biochem* 1969, **30**, 51–57.
16. Hudson L, Hay FC. *Practical Immunology*. Oxford, Blackwell, 1976, 29–39.
17. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 1959, **37**, 911–917.
18. Unger C, Damenz W, Fleer EAM, *et al.* Hexadecylphosphocholine: a new etherlipid analogue. Studies on the antineoplastic activity *in vitro* and *in vivo*. *Acta Oncologica* 1989, **28**, 213–217.
19. van Dam-Mieras MCE, Slotboom AJ, Pieterson WA, de Haas GH. The interaction of phospholipase A2 with micellar interfaces. The role of the *N*-terminal region. *Biochemistry* 1975, **14**, 5387–5394.

Acknowledgement—This study was supported in part by the Bundesministerium fuer Forschung und Technologie (BMFT).

Eur J Cancer, Vol. 29A, No. 1, pp. 101–107, 1993.
Printed in Great Britain

0964–1947/93 $5.00 + 0.00

Diversity of Human p53 Mutants Revealed by Complex Formation to SV40 T Antigen

J. Bártek, B. Vojtěšek and D.P. Lane

The products of the two major suppressor genes p53 and Rb interact with the oncogene products of the DNA tumour viruses. These viral–host protein interactions mimic and interfere with the normal interactions of p53 and Rb with host proteins. The Rb gene product is frequently mutated in human cancers such that it no longer binds to viral or host proteins. In contrast we find that this is not the case with p53 as some, but not all, mutant p53 proteins still bind to the SV40 T antigen. In particular the hot spot mutation found in most Chinese and African cases of hepatocellular carcinoma (HCC) retains T binding activity. The simple subdivision of different p53 mutations revealed by this analysis may have diagnostic and prognostic consequences.
Eur J Cancer, Vol. 29A, No. 1, pp. 101–107, 1993.

INTRODUCTION

THE ONCOGENES of the DNA tumour viruses act at least in part by physically complexing to the protein products of two tumour suppressor genes, p53 and retinoblastoma (Rb). These interactions are proposed to neutralise the growth regulatory activity of the suppressor gene products by blocking their interactions with other host proteins [1, 2] or by leading to their rapid degradation [3]. Strong support for these ideas has come from the finding that the oncogenic activity of the DNA tumour viruses is curtailed by mutations that destroy the p53 or Rb binding activity of their oncoproteins [4–6]. Furthermore the mutations of the Rb gene found in human cancer seem to be specifically localised to the sites on the Rb protein required for binding to papova virus T antigens and adenovirus E1a proteins [7–9]. The capacity of p53 to bind to the SV40 T antigen is an intrinsic property of the protein as the two purified proteins will assemble *in vitro* [10]. This activity of p53 has been conserved in evolution leading to the suggestion that the interaction mimics a normal interaction of p53 with a host protein that is essential

Correspondence to D.P. Lane.
D.P. Lane and B. Vojtěšek are at the CRC Laboratories, Department of Biochemistry, University of Dundee, Dundee DD1 4HN, U.K; and J. Bártek is at the Institute of Haematology and Blood Transfusion, Korunní 108, Prague 10, Czechoslovakia.
Recieved 13 May 1992; accepted 8 July 1992.