Original Article

Egg Yolk Phospholipids Enriched with 1-*O*-Octadecyl-2-Oleoyl-*sn*-Glycero-3-Phospho-(*N*-Palmitoyl) Ethanolamine Inhibit Development of Experimentally Induced Tumours

(hen egg phospholipids / phospholipid derivative NAEPE / inhibition of tumour cells / inhibition of liver / lung / kidney tumours / chicken model)

V. KARAFIÁT, P. VESELÝ, M. DVOŘÁK

Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, v. v. i., Prague, Czech Republic

Abstract. Dietary phospholipids (PLs) and their derivatives have proved active in suppression of various health problems and conditions including cancer. In this work we compared the effect of dietary phospholipids from hen egg yolk enriched with N-acyl ether-phosphatidyl ethanolamine (NAEPE) termed bioactive phospholipids (BAP+ preparation) with PLs lacking NAEPE (BAP– preparation) on the growth of transformed cells *in vitro* and on the promotion and progression of experimental tumours *in vivo*. For the *in vivo* experiments we used the chicken model in which liver, lung, and kidney tumours arose via natural selection from single cells initiated by experimentally introduced somatic mutations caused by insertional mutagenesis. Mutagenized animals were fed BAP+ or BAP– diet in various regimens. We observed that BAP+ at low concentrations killed cells of various tumour cell lines in culture but did not compromise viability of non-transformed cells. Oral administration of the BAP+ preparation efficiently reduced progression of all tumour types. However, it did not significantly reduce the number of already initiated tumours and their growth when BAP+ was discontinued. Our data suggest that NAEPE combined with hen egg PLs significantly interferes with tumour progression, possibly through the inhibition of tumour cell viability.

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Corresponding author: Michal Dvořák, Institute of Molecular Genetics AS CR, v. v. i., Vídeňská 1083, 142 20 Prague 4, Czech Republic. Phone: (+420) 296 443 390; Fax: (+420) 296 443 586; e-mail: mdvorak@img.cas.cz

Abbreviations: BAP – bioactive phospholipids (phospholipid fraction from hen egg yolk), CCl_4 – carbon tetrachloride, CEF – chicken embryo fibroblasts, FA – fatty acid, ICC – intrahepatal cholagiocarcinoma, MAV-2 – myeloblastosis-associated virus-2, NAEPE – N-acyl ether-phosphatidyl ethanolamine, NRK – normal rat kidney, PE – phosphatidyl ethanolamine, PL – phospholipid.

Introduction

The role and beneficial effects of dietary phospholipids (PLs) and their fatty acids (FAs) from different natural sources have been extensively studied (recently reviewed by Kuksis, 1992; Kremmyda et al., 2011; Tvrzicka et al., 2011; Kullenberg et al., 2012). Protective effects of PLs on different conditions and symptoms (e.g., inflammation, coronary heart disease, or cancer) have been mentioned in many studies (Espinosa-Salinas et al., 2011; Kullenberg et al., 2012). The PLs and their FA residues are incorporated in the plasma membranes of cells and affect their structure and physicochemical properties (Ma et al., 2004; Lev, 2010; Calder, 2012). FAs serve as substrates for biosynthesis of signalling molecules, and in consequence several cell-signalling pathways are altered which can either ameliorate or promote many pathological conditions (Farooqui and Horrocks, 2006; Khanapure et al., 2007; Krishnamoorthy and Honn, 2008; Jenkins et al., 2009). Accumulating evidence suggests that these effects significantly affect mainly rapidly proliferating cells, among them tumour cells.

Phosphatidyl ethanolamine (PE), an abundant phospholipid found in biological membranes, is taken up in common food products of plant and animal origin. The majority of PE molecules contain two acyl chains connected through an ester linkage to the glycerol *sn*-1 and *sn*-2 carbons. In addition to these standard species, cell membranes contain plasmanyl and plasmenyl PEs with the ether or vinyl ether bonds, respectively, connecting an alkyl (alkenyl) chain to the glycerol *sn*-1 carbon.

Ether phosphatidyl ethanolamine (EPE) derivatives serve various biological functions. They are engaged as structural elements of membranes and involved in the storage and release of FAs. They serve as signalling

molecules and their precursors. Specific functions comprise regulation of membrane fluidity (Hermetter et al., 1989; Paltauf, 1994) and membrane fusion (Glaser and Gross, 1994, 1995). EPEs were shown to alter cholesterol distribution in the membranes and act as antioxidants. Their presence appears to be crucial for spermatogenesis, lens development and myelination in the central nervous system (Gorgas et al., 2006; Wallner and Schmitz, 2011; Braverman and Moser, 2012; Lizard et al., 2012). Synthetic EPE derivatives (edelfosine, miltefosine, perifosine, erucylphosphocholine and erufosine) are a class of anti-tumour agents inducing growth arrest and apoptosis (for review see van Blitterswijk and Verheij, 2008; da Silva et al., 2012; van Blitterswijk and Verheij, 2013).

N-acyl phosphatidyl ethanolamines (NAPEs) are another group of PE derivatives that count among the minority of membrane phospholipids present in both prokaryotic and eukaryotic cells (Hansen et al., 2000). In addition to their structural function, NAPEs are suggested to regulate food intake (Gillum et al., 2008). Several other functions have been ascribed to NAPEs as well (for recent review see Wellner et al., 2013). Interestingly, they were found to concentrate in ischaemic tissues displaying extensive cell death (Berger et al., 2004; Janfelt et al., 2012). NAPEs also occur in the form of ether derivatives carrying *O*-alkyl and *O*-alkenyl groups connected to the *sn*-1 carbon of the glycerol moiety.

Plasmanyl-(*N*-acyl)ethanolamines (NAEPEs) isolated from ischaemic tissues of chicken embryos and semisynthetic 1-*O*-octadecyl/hexadecyl-2-oleoyl-*sn*-glycero-3-phospho-(*N*-palmitoyl)ethanolamines exhibited inhibitory effects against tumour cell lines in tissue culture. Subcutaneous injections of these ethanolamines also restricted growth of subcutaneous transplanted sarcomas in mice (Kara et al., 1986, 1993). Only few other studies addressed the potential anti-tumour effects of hen egg phospholipids (Sakakima et al., 2007, 2009).

In this work we tested the anti-tumour effect of oral administration of hen egg yolk phosholipids enriched with NAEPE in the experimental system in which liver carcinomas, lung angiosarcomas and nephroblastomas develop in outbred chickens by *in vivo* selection from single tumour cells initiated by insertional mutagenesis. Experimental tumours are thus confronted with all defence mechanisms of the body. Our study revealed a strong *in vivo* anti-tumour effect of hen egg phospholipids enriched with NAEPE, which warrants further analyses and possible finding of consequences relevant for human healthy nutrition.

Material and Methods

Phospholipids

Phospholipids (PLs) constituting the BAP– preparation were extracted from hen egg yolk with ethanol and purified using acetone precipitation according to standard procedures (Gladkowski et al., 2012). BAP+ was the BAP– preparation enriched to a final concentration of 30 % with 1-*O*-octadecyl-2-oleoyl-*sn*-glycero-3-phospho-(*N*-palmitoyl)ethanolamine as described (Vojkovsky and Liebl, 1996; Kára et al., 1997) to yield BAP+. BAP+ and BAP– preparations were prepared and provided by AREKO (AREKO Ltd., Prague, Czech Republic).

Animals, cells and virus

Brown Leghorn C/E gs^- embryos and hatched chicks from leukosis-free flocks (Hložánek and Sovová, 1968), obtained from IMG hatchery were used throughout the study. The animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals, approved by the Animal Care and Use Committee of the Academy of Sciences of the Czech Republic. Chicks were kept under standard laboratory conditions with free access to food and water.

Chicken embryo fibroblasts (CEF) (prepared from Brown Leghorn C/E gs^- embryos), chicken sarcoma cell line PR9692 (Svoboda et al., 1992), and chicken hepatocarcinoma cell line LMH (kind gift from Dr. Venugopal Nair) were cultivated in Dulbecco's modified Eagle's medium (DMEM D6171; Sigma-Aldrich, St. Louis, MO) supplemented with 8% FCS (PAA Lab. GmbH, Pasching, Austria); 2% chicken serum (Sigma), and L-glutamine / penicillin / streptomycin (G6784, Sigma). Normal rat kidney (NRK) cells and human melanoma cell line C32 (kind gift from Dr. J. Králová) were cultivated in DMEM (D6171, Sigma) supplemented with 10% FCS (PAA Lab.) and L-glutamine / penicillin / streptomycin (G6784, Sigma). All cell cultures were cultivated at 37 °C in a humidified incubator with 5% CO_2. CEF for intraembryonal inoculation were infected by a MAV-2 virus stock as described (Karafiat et al., 2001). MAV-2 was the MAV-2(N)-type virus isolated from the AMV-BAI-A complex stock by plaque purification as described (Pecenka et al., 1988a, b, c).

Tumour induction and sample collection

Twelve-day chicken embryos were inoculated via the chorioallantoic vein with 1×10^5 MAV-2-producing cells as described (Pajer et al., 2006). Carbon tetrachloride (CCl_4) (Sigma), 20% solution in olive oil, was injected intraperitoneally (2 µl/g of animal weight) 8, 12, and 16 days post hatching. The animals were sacrificed and examined 6 to 10 weeks post hatching. Macroscopically discernible tumour lesions were resected for histological analysis.

Application of phospholipids

Suspensions of both BAP+ and BAP– preparations were administered to fasting animals into the pharynx ("oral" administration) with the help of a syringe, 0.15 ml/20 g of animal weight. The animals were fasting before administration of samples, so that the solutions passed through directly into the stomach. The samples were administered from day 7 post hatching daily in the course of two weeks, and then every other day until the end of the experiment.

Histological investigation

For histopathological evaluation, harvested tumours were fixed in 4% buffered paraformaldehyde, embedded in paraffin blocks, and processed by routine histological procedures with haematoxylin and eosin staining. The stained sections were examined.

BAP treatment of cell cultures in vitro

The cells were plated on 30-mm dishes and left to adhere. Then, the cultivation medium with 0.1% BAP+ or BAP– was added and the cells were kept for additional 10 days. Cells were then fixed and stained with May-Grünwald-Giemsa.

Statistical analysis

Analysis of the tumour incidence was performed using Pearson's χ^2 test with Yates' continuity correction, and Fisher's exact test. Binominal confidence intervals for samples were computed.

Image acquisition and processing

Images were obtained with a Leica DMIRB microscope (Leica Microsystems GmbH, Wetzlar, Germany) and processed using Adobe software (Adobe Systems, San Jose, CA).

Results

Effects of BAP+ on tumour cells in vitro

To examine the effect of BAP *in vitro*, both normal avian CEF and mammalian NRK cells and the tumour cell lines – avian PR9692 and LMH and human C32 – were treated with 0.1% concentration of BAP+ or BAP– for 10 days. BAP+ efficiently impaired the viability of tumour cells and induced their death. The normal cells treated with BAP+ survived without changes in viability. Control BAP– had no significant effect on the viability of either cell type (Fig. 1).

Oral intake of BAP+ reduces formation of various tumours in experimental animals

High incidence of tumours was induced in chickens by the combination of industasis (Pajer et al., 2009) and chemical carcinogenesis. MAV-2- producing mesenchymal cells were inoculated by intravenous injection into 12-day-old chicken embryos (Pajer et al., 2009). Carbon tetrachloride, CCl_4 (20% solution in olive oil), was injected intraperitoneally into animals in three doses post hatching. The animals were divided randomly into three groups. The first and second groups received either BAP+ or BAP– doses from day 7 post hatching daily in the course of two weeks, and then three times a week until the age of 44 days, when the animals were sacrificed and analysed. These groups were termed BAP+ (7-44/44) (the group included 19 animals, N = 19) and BAP– (7-44/44) (N = 22). The third group received no treatment. Since no difference was observed between the groups receiving BAP– and untreated animals, only BAP– data are shown. The experimental design is depicted in Fig. 2a.

Tumours were found in the lungs, livers, and kidneys. Some animals carried tumours in two or three of these organs simultaneously (Table 1).

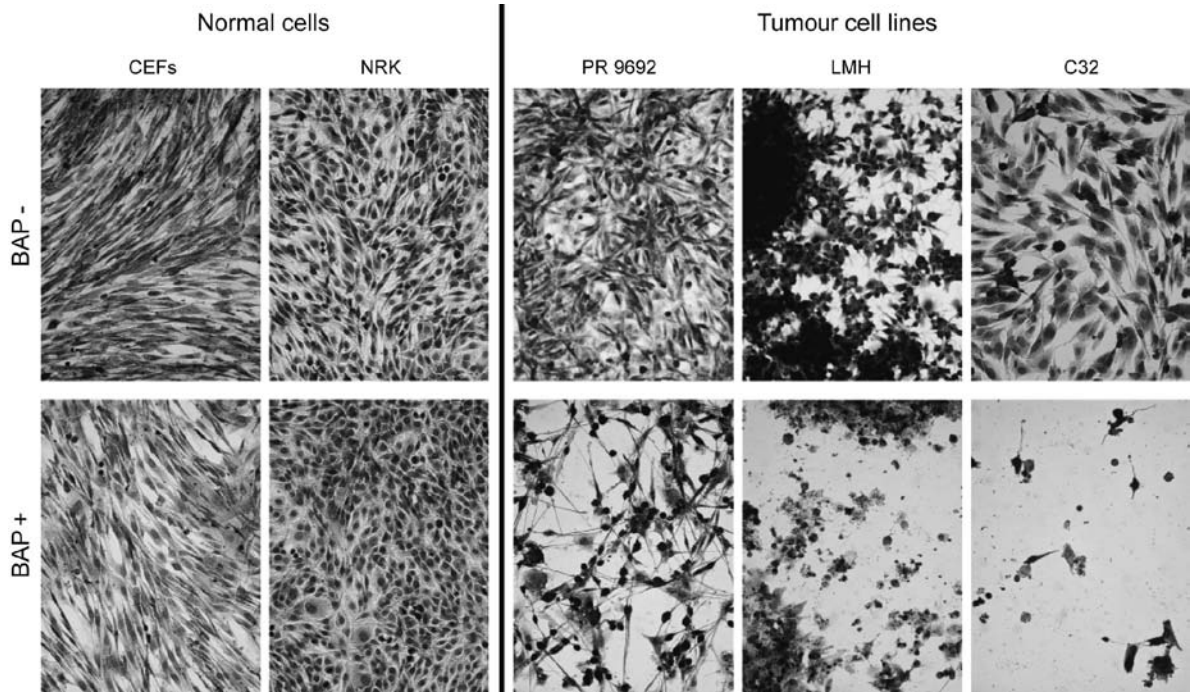

Fig. 1. Effects of BAP+ on tumour cells in culture. The normal avian (CEFs) and mammalian (NRK) cells, and the tumour cell lines (avian PR9692 and LMH, and human C32) treated with control BAP– (upper row) and BAP+ (lower row) preparations.

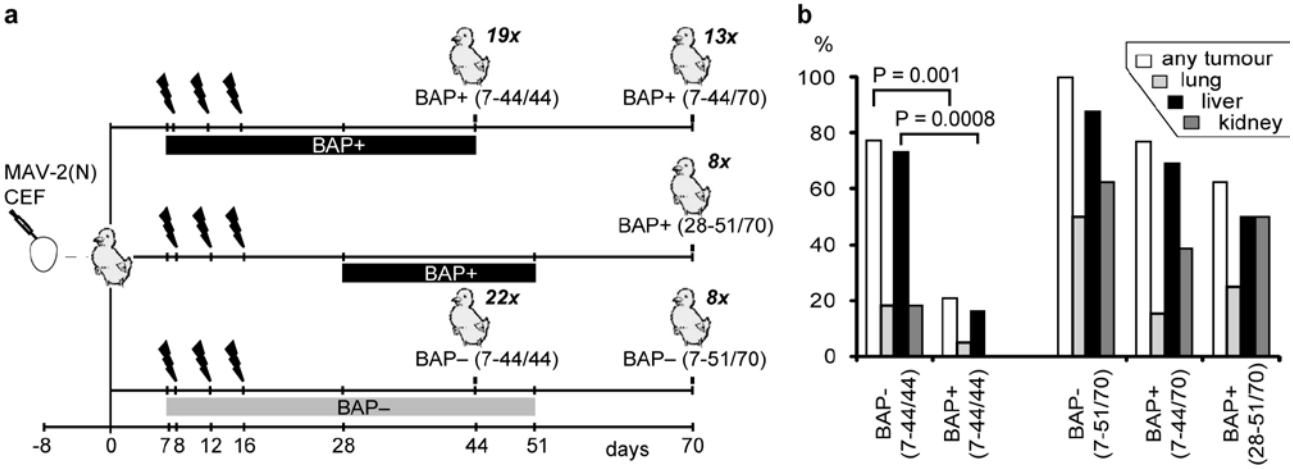

Fig. 2. (**a**) Experimental design; detailed information is given in the text and in Material and Methods. Lightning bolts mark days of CCl_4 administration. (**b**) Tumour incidence in experimental animals; the proportion of chickens carrying tumours in different organs is shown. The type of phospholipid treatment is displayed on the x-axis. Significance was assigned at $P < 0.05$.

The results are summarized in Fig. 2b. Animals receiving BAP+ showed a significantly lower incidence of observable tumours when compared with control animals receiving BAP–. While 77 % (17/22) of BAP– (7-44/44) animals developed tumours, tumours were observed only in 21 % (4/19) of BAP+ (7-44/44) animals. The decrease in tumour incidence caused by BAP+ was statistically significant with the P value = 0.0010. Among BAP– chickens, 73 % (16/22) carried liver tumours, 18 % (4/22) lung tumours and 18 % (4/22) nephroblastomas. Among BAP+ (7-44/44) chickens, 16 % (3/19) developed liver tumours and 5 % (1/19) lung tumours. No nephroblastoma was observed. Two independent experiments were performed, and the numbers represent cumulative values from both experiments.

Since tumour cells are initiated within the first few days after MAV2 inoculation and subsequent initiations are very rare (Pajer et al., 2009), it is possible to find out whether BAP+ treatment killed tumours already in initial stages or only blocked their progression. In order to address this question, the following experiments were performed:

a) animals mutagenized and CCl_4-treated in a standard way received the first BAP+ dose 28 days post hatching (when the first macroscopic tumours began to appear (Pajer et al., 2009; Pecenka, unpublished observations) and the animals kept receiving BAP+ until the age of 51 days. At the age of 70 days, the animals were sacrificed and examined – the group BAP+ (28-51/70) (N = 8). Tumours were found in 63 % (5/8) animals – liver tumours in 50 % (4/8) cases, lung tumours in 25 % (2/8) cases, and kidney tumours in 50 % (4/8) of animals. On the other hand, 100 % (8/8) of BAP– animals examined 70 days after hatching – group BAP– (7-51/70) (N = 8) – carried tumours; liver tumours were found in 88 % (7/8) cases, lung tumours in 50 % (4/8) cases, and kidney tumours in 63 % (5/8) of animals.
b) animals mutagenized and CCl_4-treated in a standard way were receiving BAP+ until day 41 but were examined 20–30 days later – group BAP+ (7-44/70) (N = 13). Tumours were found in 77 % (10/13) animals, while only 23 % (3/13) BAP+ animals examined on day 44 presented tumours. Tumour incidence in the livers, lungs and kidneys in the BAP+ (7-44/70) group was 69 % (9/13), 15 % (2/13), and 38 % (5/13), respectively. Since the tumours were initiated before hatching and CCl_4-promoted until about 20 days after hatching, the results of the experimental setting b) show that the tumours exist in the latent form and their growth is restricted by BAP+ as long as the preparation is administered. This is also supported by the results of a). A slight reduction of tumour incidence was observed for both BAP+ (28-51/70) and BAP+ (7-44/70) treatment, although it did not reach statistical significance (P values > 0.05) due to the small number of animals in these two groups.

Table 1. The number of animals having tumour in one or more organs (liver, lung, kidney)

Treatment	No. of animals with tumours		
	1 organ	2 organs	3 organs
BAP– (7-44/44)	12	3	2
BAP– (7-51/70)	1	5	2
BAP+ (7-44/44)	4	0	0
BAP+ (7-44/70)	6	3	1
BAP+ (28-51/70)	1	2	2

Evaluation of tumours from BAP+ and BAP– – treated animals

Tumour lesions in the lungs, livers, and kidneys were analysed by gross morphology and histology (Fig. 3). Kidney tumours (nephroblastomas) found in the animals from groups BAP+ (7-44/70) and BAP+ (28-51/70)

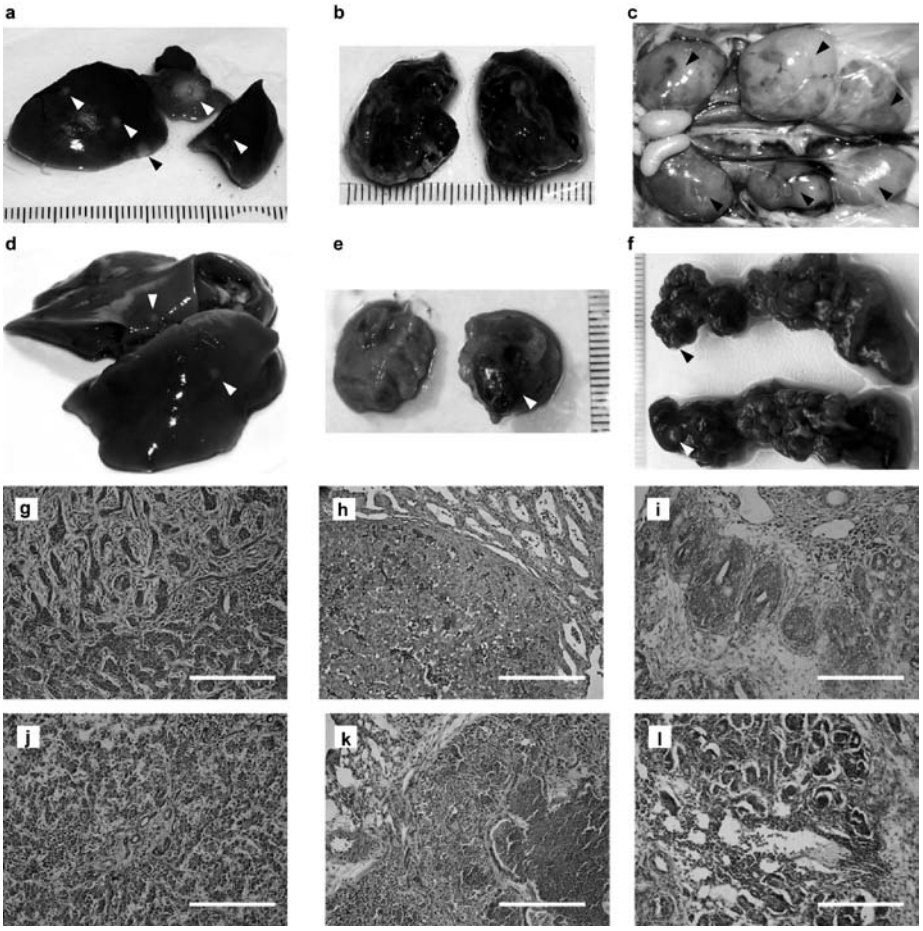

Fig. 3. Gross morphology (arrowheads) (**a** – **f**) and histology (**g** – **l**) of tumours; examples of tumours representing different size categories are shown. (**a**, **g**) Liver tumours of a control BAP- animal (large ICC with rich amounts of fibrous stroma); (**d**, **j**) small ICC focus from a BAP+ animal; (**b**, **h**) generalized tumours in both lungs of a control BAP- animal; (**e**, **k**) distinct focus in the lung of a BAP+ animal; (**c**, **i**) large kidney tumours (stage III) of a control BAP- animal; (**f**, **l**) small kidney tumours (stage I) of a BAP+ (7-44/70) animal. Bars represent 200 μm.

fell into stage I – II, in contrast to the tumours in BAP– (7-44/44 and 7-51/70) animals that fell into stage I – III according to the proportions of typical histopathologic attributes (nests of abnormal nephrogenesis, cystic dilations of tubules, and growing proportion of unorganized cells) and based on qualitative and quantitative representations of these structures (stage 0 – III) (Pajer et al., 2006). Lung tumours were angiosarcomas (Pajer et al., 2009). The BAP– animals developed either distinct foci or generalized aggressive tumour lesions, in a few cases afflicting both lungs. On the other hand, only distinct foci were apparent in the lungs of BAP+ (28-51/70) and BAP+ (7-44/70) chicks, and rarely in BAP+ (7-44/44) animals. The liver tumours were the most numerous in the tumour-bearing animals. The most prevalent liver tumours were intrahepatal cholagiocarcinomas (ICC) (Table 2). We also monitored the size of tumour foci and their number per one liver in BAP+ and control BAP– animals. Note that the number of tumours in the group BAP+ (7-44/44) was very small and does not enable statistical analysis. The size of tumours that developed after the entire treatment in BAP+ animals was not significantly restricted within the group BAP+ (7-44/44) (Fig. 4a). The rather large tumours (diameter 5 mm) were found in two animals and represent exceptional cases. Tumour foci greater than 5 mm (up to 1.5 cm) were detected in BAP– animals only. No significant differences in the number of tumour foci per liver were observed (Fig. 4b). Two tumour foci per liver were the most fre-

Table 2. The number of liver tumours of different histotypes. Cholangiocarcinoma (ICC) was the most prevalent histotype. Hepatocellular carcinoma (HCC), haemangiosarcoma (HHS), and combined hepato-cholangiocarcinoma (ICC/HCC) were only rare cases

Treatment	Histology			
	ICC	ICC/HCC	HCC	HHS
BAP– (7-44/44)	40/43[+)]	2/43	1/43	0
BAP– (7-51/70)	16/17	1/17	0	0
BAP+ (7-44/44)	5/5	0	0	0
BAP+ (7-44/70)	12/13	0	0	1/13
BAP+ (28-51/70)	9/10	1/10	0	0

[+)] number of tumours of a particular histotype/number of all tumour foci

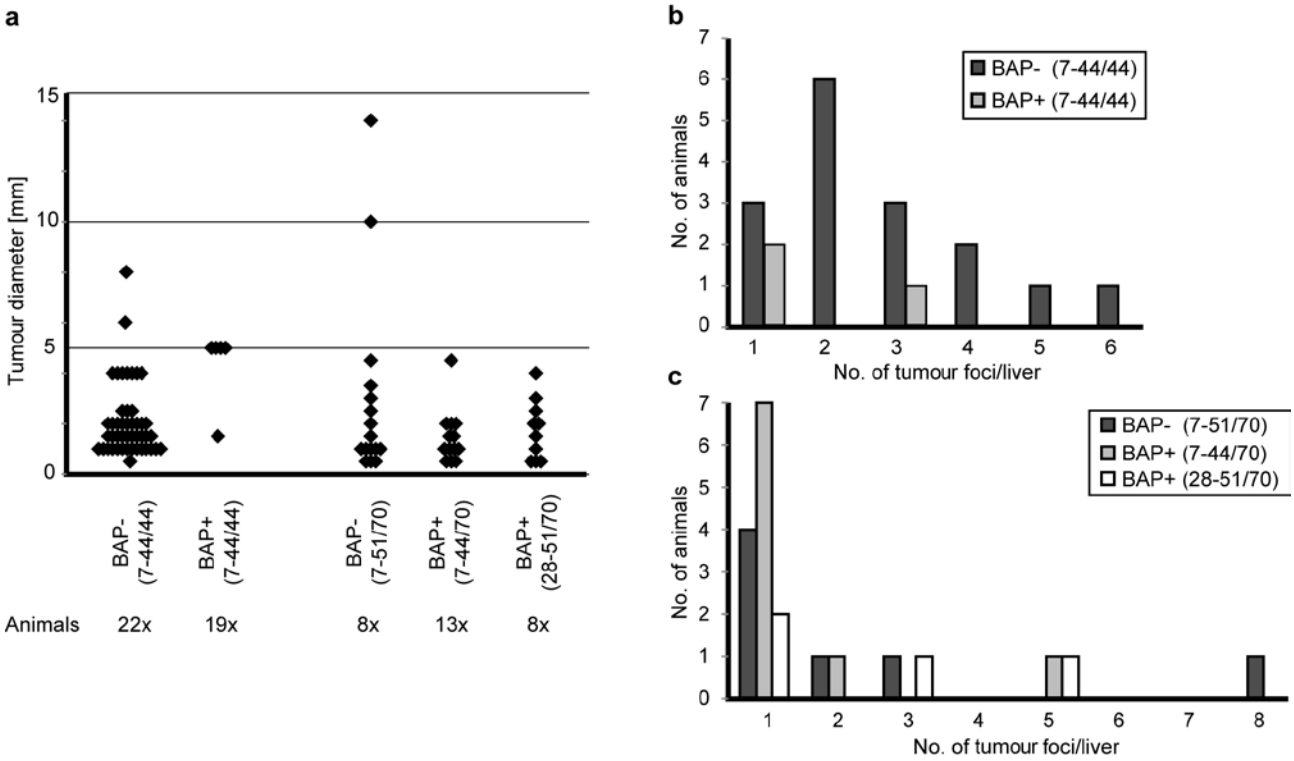

Fig. 4. Liver tumours. (**a**) Size of liver tumours; the relationship between size (y-axis) of individual tumour foci and type of phospholipid treatment (x-axis). The number of animals in each group is given. (**b**) Number of tumour foci per liver at the age of 44 days. (**c**) Number of tumours per liver at the age of 70 days.

quent in BAP– (7-44/44) animals and the highest number of tumour foci per liver in this group was 6. Three tumour foci per liver were found in a single BAP+ (7-44/44) animal only and one tumour focus occurred in other two animals in this group. The highest numbers of tumour foci per liver in BAP+ (28-51/70) and BAP+ (7-44/70) groups and in the BAP– (7-44/70) group were 5 and 8, respectively.

Discussion

The chicken model has been used in the study of a broad variety of human diseases (Batini et al., 2004; Rashidi and Sottile, 2009; Smith and Chan, 2010; Datar and Bhonde, 2011; Johnson and Giles, 2013). We have developed a chicken model of carcinogenesis based on insertional mutagenesis by myeloblastosis-associated virus-2 (MAV-2) (Pajer et al., 2003, 2006, 2009). Infection of chickens with MAV-2 leads to the formation of three tumour types: nephroblastomas, liver carcinomas, and lung angiosarcomas. Integration of proviral DNA into host chromosomes induces tumours by activating the tumorigenic potential of cellular oncogenes or by inactivating cellular tumour suppressor genes (Pecenka et al., 2011). In this model, tumour cells are derived from the cells of the body, which are under regular control of tissue homeostasis, and must go through selection similarly as naturally arising tumours. We have recently reported (Pecenka et al., 2011) that the formation of liver tumours in this chicken model is the consequence of the mutation and activation of c-*Ha-ras,* c-*erbB/EGFR,* c-*ron*/c-*stk*/c-*sea/MST1R* or c-*met/HGFR*, i.e. the same genes implicated in human liver tumours (Olayioye et al., 2000; Gentile et al., 2008). Moreover, detailed histological examination enabled classification of chicken liver tumours to categories matching human liver tumours (Weiss et al., 2007; International Consensus Group for Hepatocellular Neoplasia, 2009; Nakanuma et al., 2010).

Since the metabolism of dietary lipids in chickens is similar to that in mammals (Griffin et al., 1989; Hermier, 1997), the chicken model we used provided relevant information suggesting that hen egg phospholipids might also inhibit the progression of human liver tumours.

There are only limited studies aimed at analysing potential anti-tumour effects of hen egg phospholipids. In this work we used phospholipids enriched with NAEPE, which was present in the preparations tested earlier (Kara et al., 1986, 1993). The mixture of NAEPE and PLs was used because NAEPE purified according to the described procedure and orally administered was less active than BAP+ (Pouckova, unpublished observations). We suggest that it might be either due to the supportive effect of other PLs in resorption and transport, or to their supportive effect in the membranes of cancer cells. The *in vitro* experiments strongly suggest that BAP+ compromises the viability of transformed cells. However, it does not seem to eliminate fully initiated cancer cells *in vivo* – only the progression of tumours is blocked. In conclusion, it was observed that administra-

tion of the BAP+ preparation efficiently reduced progression of liver, lung, and kidney tumours *in vivo* and compromised the *in vitro* viability of randomly selected tumour cell lines. Therefore, BAP+ does not seem to display any selectivity for the type of tumour cells. We suggest that BAP+ interferes with tumour progression through partial inhibition of tumour cell viability, which is supported by the *in vitro* experiments. The presented chicken cancerogenesis model has a potential for testing bioactive food compounds in relation to cancer.

Acknowledgements

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