

## ANTINEOPLASTONS IN DAIRY PRODUCTS

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The compounds isolated by our team from plasma and urine, and named antineoplastons (ANP), decrease expression of oncogenes and activate silenced tumor suppressors. Four ANP ingredients, 3-phenylacetyl-amino-2, 6-piperidinedione (A10), phenylacetylglutamine (PG), phenylacetylglutamine (isoPG), phenylacetic acid (PN) and one pro drug, 4-phenylbutyric acid (PB), were determined in milk, farmer's and feta cheese and whey by a Shimadzu HPLC system. PG and isoPG occur in the highest concentrations in whey (29.0 and 6.0 mg/100 mL) and farmer's cheese (22.0 and 3.0 mg/100 g). A10 and PN were found in the highest concentration of 7.0 mg/100g and 4.0 mg/100g correspondingly in farmer's cheese. PB occurred in trace amounts in farmer's cheese. In conclusion, A10, PG, isoPG, PN and PB exist in small amounts in dairy products. Based on the previous studies, the supplements containing ingredients of antineoplastons may play an important part in prevention of cancer and anti-aging.

**Keywords:** Antineoplastons in food, chemoprevention, anti-aging, phenylacetate, phenylbutyrate, phenylacetylglutamine.

### INTRODUCTION

The first few years of the new century coincided with the 100<sup>th</sup> anniversary of radiation therapy and the introduction of new, exciting molecular-targeted therapies for cancer treatment (1, 2). Prominent oncologists believe that this is the end of conventional cancer treatment and the beginning of a new era of molecular therapies. The agents of targeted therapies, such as monoclonal antibodies or small molecules, correct the genetic changes which trigger the cancerous process, with only minimal adverse

gene expression by "turning off" overexpressed oncogenes and "turning on" silenced tumor suppressor genes (5-7). Study of the human genome revealed that only about 10% of our genes are active at any time in adult life (8). The genes are being turned on and off by the system of biochemical factors named epigenome (9,10). In case of a malfunction of this system, the genes which promote cancer—oncogenes—will be over-expressed and the genes, that protect against cancer—tumor suppressor genes—will be silenced. A disturbed balance in the expression of these genes leads to cancer (8). Silencing of important genes, such as WRN1 helicase, will lead to premature aging as documented in Werner syndrome (11). Cancer

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Burzynski Clinic, 9432 Old Katy Road, Houston, Texas 77055, phone # (713) 335-5697, fax # (713) 335-5658, reactions (3, 4). A similar approach is reflected in cancer prevention, with the introduction of the new generation of food supplements effecting

and aging are integral parts of life and can be traced to very primitive organisms. The most important genes and "molecular switches" involved in these processes can also be traced from humans to rodents, insects, worms, plants, and even yeast (12).

The proper use of these molecules in our diet can provide us with a defense against cancer and slow down genetic aging. These substances, isolated from plants, prevent cancer by turning off the signal in important oncogene pathways (6). The chemicals of human origin, which also were isolated from animal products, play an important part in reducing the activity of over-expressed oncogenes and in activation of silenced tumor suppressor genes (8, 13). Our team has isolated these compounds from human blood and urine since 1968 and named them antineoplastons (ANP) (13, 14). The emphasis was placed on ANP which were reproduced synthetically, including 3-phenylacetyl-amino-2,6-piperidinedione (A10) (15), phenylacetylglutamine (PG) (16) and phenylacetic acid (PN) (17).

Early research data indicated that ANP are species-specific. It was found, initially, that the only animal species, in addition to humans, which conjugate substantial quantities of L-glutamine with phenylacetic acid (the first step in the synthesis of A10) are old world monkeys (18-23). The other animal species conjugate different amino acids with phenylacetic acid: mice-glycine, rats and dogs-L-glutamic acid, and ferrets-aurine (21-23). Sheep are an exception; they produce 4-phenylbutyric acid (PB), which, in the human liver, converts into PN and PG (24, 25). Studies performed by researchers from the USDA revealed that PG is a normal ingredient of cows' milk, raising the possibility that other antineoplastons and their prodrug (PB) may also exist in small quantities in dairy products (26).

The objective of this study is a quantitative determination of A10, PG, phenylacetylglutamine (isoPG), PN, and PB in dairy products.

## MATERIALS AND METHODS

### Materials

Farmer's cheese, feta cheese, fresh whole milk and condensed milk are commercially available products obtained from the store.

Curd and whey were prepared in our laboratory according to the procedure described below.

### Sample preparation

#### Farmer's cheese and feta cheese

The portion (approx. 40-60g) of raw farmer's or feta cheese was homogenized using Biohomogenizer model M113 (Biospec Products Inc.). The homogenized sample was weighted and then packed in extraction thimbles made of filter paper (Whatman No 4). Extraction was carried out in the Soxhlet apparatus, with methanol as the solvent, for approx. 25 hrs. 100 mL of methanol (HPLC grade, Fisher Scientific) was used for each extraction. When the extraction process was finished, methanol was partially evaporated from the extract on the rotary evaporator RE47 (Yamato Scientific Co. Ltd.). Then, a small amount of water was added to the mixture to enable freezing of the solution. The condensed extract was frozen, then lyophilized to dryness using a VIRTIS freeze dryer (The Virtis Company, Gardiner, N.J.). The process was carried out for approx. 48 hrs. Next, the dry residue was reconstituted with 1 mL of N,N-dimethylformamide (DMF) (HPLC grade, Fisher Scientific). Samples varied from one another, so, for some of them, 1 mL of the solvent was not enough. The dry residue extensively absorbed the solvent. In such cases more DMF had to be used. Due to the higher amount of dry residue, the feta cheese extract was reconstituted with 2 mL of DMF. In some experiments, DMF was replaced by methanol, water: methanol mixture (1:5), 0.01M sodium hydroxide (NaOH) or methanol: 0.01M NaOH mixture (5:1). Generally, changing the solvents did

not improve the solubility of the dry residue. The mixture was centrifuged for 20 min. at 13,000 rpm in order to separate insoluble ingredients from the extract. Some part always remained insoluble. Then the solution was filtered through a 0.45 $\mu$ m nylon syringe filter (Whatman).

#### **Milk and condensed milk**

100 mL of whole fresh or condensed milk was frozen. Then it was lyophilized to dryness using a VIRTIS freeze dryer. The dry sample was treated similarly to farmer's cheese.

#### **Curd and whey.**

A portion of whole fresh milk (1.8 L) was warmed up to 28°C. It was stirred well and ½ enzymatic tablet (rennet) dissolved in 100 mL of distilled water was added to the warm milk. The mixture was stirred well for 15 min. Then it was left aside for 2 hrs at room temperature. Next, the formed curd was cut and drained through the Whatman paper filter No 2. The separated whey and curd were taken for further analyses. The whey was condensed on the rotary evaporator (approx. 10x) and 120 mL of concentrated whey was lyophilized. The dry residue was then loaded into the Soxhlet apparatus and the extraction process was carried out according to the described procedure.

The extraction was preformed in three replications for each sample. Additionally, the blank extraction was performed in each series.

#### **Preparation of standard solution**

A stock solution of antineoplaston standards was prepared by dissolving 0.100 g of each compound in 10 mL of DMF. Then, the standard solutions used for the calibration were prepared by diluting the stock solution with DMF to obtain the target concentrations of: 2.0 mg/mL, 1.0 mg/mL and 0.5 mg/mL.

#### **HPLC analysis**

HPLC was carried out using a Shimadzu HPLC system containing two LC-AS pumps, an SCL-10A controller, SIL-10A autoinjector, SUS variable volume injector

and SPD-10A UV detector. All chromatography buffers were made with HPLC water, filtered through a 0.2 $\mu$ m hydrophilic filter (Millipore) and degassed under vacuum. The reversed-phase column,  $\mu$  Bondpak C<sub>18</sub> (Waters) had dimensions of 300 x 3.9 mm I.D. and 10  $\mu$ m particle size. The mobile phase was water: methanol: acetic acid (78:22:1 v/v/v) during the first 70 minutes and further was in a 59:41:1 (v/v/v) ratio. 45 $\mu$ L of the sample was injected into the HPLC and separated at 0.9 mL/min at a temperature of 27°C. The analyzed compounds were detected at 254 nm. ANP levels, expressed in mg/mL, were calculated from the peak areas, using the EZ-Chrom Data System software from Shimadzu. The actual concentrations of antineoplastons in the particular dairy products were recalculated, considering the weight of the specimen taken for extraction as well as the final volume of the reconstructed solution.

## **RESULTS**

The detection of PG in cow's milk (26) inspired us to study the other ANP components, i.e. A10, IsoPG, PN and the ANP prodrug PB. Several samples of farmer's cheese, originating from various batches, were studied in order to determine quantitatively the presence of the compounds. It was found that PG is the most abundant ANP component not only in farmer's cheese, but also in all studied dairy products. Nevertheless, the results obtained varied significantly. The highest concentration of PG was calculated as 22.0 mg/100g (average of three replicates), while the lowest concentration was 1.0 mg/100g. Moreover, the chromatograms differed significantly, suggesting that the specimens from different batches were not uniform. The typical chromatogram is presented in the Figure 1.

The other compounds, i.e. isoPG, A10 and

TABLE 1. Antineoplastons in Dairy Products

	MILK (mg/100mL)	CONDENSED MILK (mg/100mL)	FARMER'S CHEESE (mg/100g)	FETA CHEESE (mg/100g)	WHEY (mg/100mL)
Phenylacetylglutamine (PG)	6.0	18.0	22.0	3.0	29.0
Phenylacetylisoglutamine (isoPG)	0.1	0.1	3.0	1.0	6.0
3-phenylacetylamino-2,6- piperidinedione (A10)	0.1	0.1	7.0	0.1	3.0
Phenylacetic acid (PN)	Not found	Not found	4.0	Not found	0.3
4-phenylbutyric acid (PB)	Not found	Not found	traces	Not found	Not found

The results are averages from 3 analyses

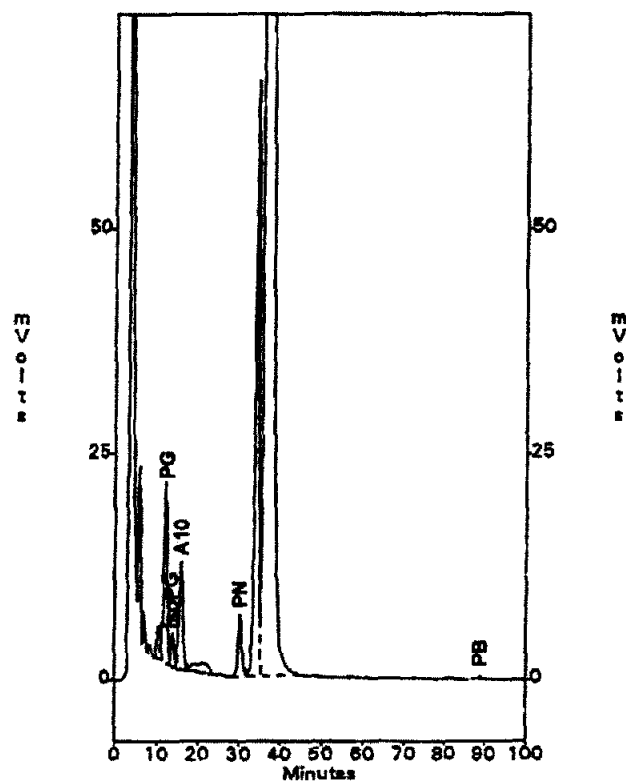


FIGURE 1. Typical HPLC chromatogram of antineoplaston ingredients in farmer's cheese. A10—3-phenylacetylamino-2,6-piperidinedione, PB—4-phenylbutyric acid, isoPG—phenylacetylisoglutamine, PG—phenylacetylglutamine, PN—phenylacetic acid.

PN were also found in farmer's cheese, but PB was not present. Their concentrations, however, were significantly lower (approx. 3.0-4.0 mg/100g) and did not vary so drastically between batches, as was the case of PG. Some analyses indicated trace amounts of PB, but the concentrations (less than a milligram per 100 g) were at the border of method sensitivity. The results are summarized in Table 1.

Our experiments confirmed the results of earlier studies on PG in cow's milk<sup>(26)</sup>. In our studies we used fresh whole milk as well as condensed milk. We found that, similar to the farmer's cheese samples, milk contained mostly PG. The concentration of PG was approximately 3 times higher in the condensed milk than in the fresh milk. The differences in the PG concentrations can be explained simply by the more concentrated sample. The other compounds, i.e. isoPG and A10 were found in trace concentrations (less than a milligram per 100 g). Nevertheless, PN and PB were not found at all (see Table 1). The significantly lower concentrations of isoPG, and A10 can be caused by the process of the milk preparation, such as pasteurization.

Interesting results were obtained in the experiment with whey. The amount of PG determined in the whey was comparable to that present in farmer's cheese. These results were obtained for the whey sample, which was concentrated on the rotary evaporator and then deproteinized by addition of acetonitrile. The other compounds, isoPG, A10 and PN were found from trace amounts to 6.0mg/100g, comparable to those present in the other dairy products.

The analysis of feta cheese was more difficult to perform due to the high content of fat. PG was in concentrations approximately 7 times lower than in farmer's cheese. This may be explained by the different source of the product (sheep vs. cow) and the loss of some compounds during the preparation steps, especially during the fat extraction.

## DISCUSSION

A10 is present in human blood and urine (14, 27). Based on the findings described in this article it also exists, in small amounts, in farmer's cheese and whey and in trace amounts in cow's milk and feta cheese. A10 specifically intercalates with DNA and protects the sequences which are vulnerable to the effects of carcinogens, such as benzo [a] pyrene, urethane, and aflatoxin B<sub>1</sub> (28, 29). In animal tests, conducted at the Medical College of Georgia, University of Kurume Medical School in Japan and Burzynski Research Institute, mice and rats were protected from development of breast, lung and liver cancers when they were exposed to carcinogens and were fed a diet containing A10 (30-34). Human pharmacokinetics studies revealed that 70% of A10 is absorbed intact from the small intestine, but 30% is converted into PG and isoPG (35). In addition, PG is biosynthesized in the liver from glutamine and phenylacetate (22, 36). PG was detected in cow's milk by researchers from the USDA and by our team (as described in this paper) in milk, farmer's cheese, whey, and feta cheese (26). It is also derived from PB, which is present in lamb (25). PG exhibits antineoplastic activity across a wide array of cancer cell lines, including breast, liver and glioblastoma multiforme (37, 38). It inhibits the uptake of growth-critical amino acids such as L-glutamine and L-leucine in neoplastic cells (38). PG enters cells by the stereospecific amino acid transporters and works as a competitive inhibitor of these transporters. It also normalizes the pattern of genome-wide methylation, stabilizes the genes, decreases expression of oncogenes and promotes apoptosis (38).

PN is present in relatively low concentration in human blood, urine, and in dairy products (39, 40). It is produced by normal intestinal bacterial flora and is generated from metabolism of PB (25, 39). It works as a molecular switch, which turns off the electrical signal in one of the

most important oncogene pathways, the *RAS* oncogenes, and activates the tumor suppressor genes *TP53* and *p210* (41-44). PN inhibits farnesylation of the p21<sup>ras</sup> protein and causes down-regulation of *BCL-2* through inhibition of mevalonate 5-pyrophosphate decarboxylase (41, 43, 45). PN activates the *TP53* and *p21* tumor suppressor genes through inhibition of methyltransferases (42, 44). It also binds excessive amounts of L-glutamine, a promoter of cancer growth (46).

PB exists in lamb and in trace amounts in farmer cheese and it is partially converted in the liver to PN and PG (24, 25). It activates tumor suppressor genes through inhibition of histone deacetylase (47).

A10 and PG were formulated together with selected amino acids and vitamin B-2 into two supplements currently available in the United States and in the European Union (48). In addition to cancer preventive effects, which were documented in animals, there have also been a number of other positive effects observed by users. Among these were anti-aging effects reported by individuals who take these supplements. Positive effects included increased energy, improved healing, reduction of wrinkles and hyperpigmentation spots, reduction of cholesterol concentration in blood, improved cellular immunity (with a decreased frequency of common cold and viral infections), improvement of benign prostate hypertrophy and a decrease of benign nodules in breasts, as well as an anti-depressant effect (48).

### CONCLUSIONS

The ingredients of antineoplastons: A10, PG, isoPG, and PN and the pro-drug PB were found in dairy products. PG occurs in the highest concentration of 29 mg/100 mL in whey, 6.0 mg/100 mL in milk, 22.0 mg/100 g in farmer's cheese and 3.0 mg/100 g in feta cheese. The other compounds were present in much smaller amounts (from trace to 7.0 mg/100 g) and these

varied significantly between samples. A10 and PN were found in the highest concentration of 7.0 mg/100 g and 4.0 mg/100 g correspondingly in farmer's cheese. PB occurred only in trace amounts in farmer's cheese.

Prevention of cancer and age management by phytochemicals and non-nutritional components of the diet is now considered to be an inexpensive and acceptable practice. The system of small molecules, such as polyphenols in plants and amino acid derivatives, carboxylic acids, and peptides in animal products apparently protect various organisms from formation of cancer and has an impact on aging. Our research group postulated the existence of such a system in 1976 (49). As early as 1980, the NCI's Chemoprevention Programme began evaluating phytochemicals for safety and efficacy. In 1998, NCI's Division of Cancer Prevention started the Chemoprevention Implementation Group. NCI has more than 400 potential agents under investigation and is sponsoring over 60 chemoprevention trials (6). The European Union, through its EPIC program (European Prospective Investigation into Cancer and Nutrition), conducts larger and more ambitious investigations (7). Over 500,000 volunteers in ten European countries are included in the studies, which link dietary, biochemical, and genetic analysis. The chemopreventive effects of most dietary products are the sum of several distinct mechanisms. In many cases the concentration of active chemicals in food is not sufficient, which is typical for substances derived from animal products. Therefore, it will be necessary to take supplements in addition to a proper diet. The new term "neutrigenomics" has been introduced, with more and more attention devoted to food supplements, which affect the genes. It is expected that, in the near future, designer foods will be available containing a chemopreventive and anti-aging agents and they will have a substantial impact on reducing the incidence of cancer.

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