A Study Regarding Boron Metabolism at Experimental Animals in Order to Achieve Neutron Irradiation in Boron Capture

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In order to obtain a first experimental model of BNCT irradiation on experimental animals in Romania we made some experiments in order to characterize boron metabolism. After the first laboratory study regarding BPA use in cancer radiotherapy via neutron capture were assessed the following issues: BPA is a substance with a limited degree of solubility in aqueous solvents. Our results allow the selection of conditioned aqueous systems which provides an increased BPA solubility degree, ranging between 30-40 μg / mL allowing the saturation with BPA in the tumor cell in order that neutron capture reaction to take place and release α particles with high linear energy transfer, effective in the therapy; BPA is a molecule with affinity for HRS1 type tumor cells being captured and accumulated at maximum values in the range 3-5 h after intravenous injection; the BPA internalization degree in HUH-7-17 type tumor cells depends on BPA concentration, a concentration of 20-40 μg BPA for about 10^5-10^6 tumor cells ensures a maximum internalization degree.

Keywords:boron metabolism, liver cancer, experimental animals, irradiation

L-p-Borono-phenylalanine (BPA) is one of the ^{10}B bearing molecules, very attractive for cancer therapy studies using BNCT (irradiation with boron neutron capture) method:

- the specificity of B carrying molecule;
- the therapeutic dose administered for which a maximum boron accumulation in the tumor cells exists;
- neutron capture reaction rate, ^{10}B (n, *a*) during ^{7}Li irradiation.

Experimental work undertaken followed the assessing of following aspects:

- the degree of BPA solubility in various solvents;
- the BPA biodistribution in liver tumor Hepatoma RS1 (HRS1) type by *in vivo* studies;
-the BPA internalization into liver tumor cells by means of *in vitro* studies;
- the cytotoxic effects using *ex vivo* studies.

Experimental part

Raw materials, materials, reagents and equipments used in order to perform the studies were:

- L-p-Borono-phenylalanine (BPA) with boron enriched 99.7% with ^{10}B imported from Katchem L, Czech Republic;
- sodium radioiodide (^{131}I) imported from Nordion, Canada;
- London Wistar rats, male, HRS1 tumor type bearing (animal models obtained in the Bucharest Institute of Oncology);
- HUH liver tumor cells (*in vitro* models made at Fundeni Clinical Institute).

The equipment used were:

- speakers and enclosures for radiochemistry works;
- radio chromatograph;
- carbon dioxide incubator - Medter Einrichtungen GmbH;
- Robotron radioisotope calibrator;
- counter gama – Spectroscaler;
- Ependorf micropipettes;
- ultracentrifuge - High Speed Centrifuge, Type 310;
- basement with air in laminar flow.

BPA solubility

Considering the literature data showing that BPA is a sparingly soluble or low solubility degree substance in aqueous solvents [1-6], we performed a study regarding BPA solubility increasing depending on: composition, ionic strength and dissolution system polarity.

They were made the following dissolution systems:

a. water - control and evaluation system;

b. water: ethanolamine: HCl 5N in the following proportion 1: 0.01: 0.05 (m / m);

c. water: triethanolamine: HCl 5N in the ratio of 1: 0.01: 0.05 (m / m);

d. phosphate buffer with *p*H=7.4 having the composition:

KH_2PO_4 0.01 M;
Na_2HPO_4 0.01 M;
KCl 2 g/L;
NaCl 8.7 g/L.

e. 50 mM glucose: 50 mM NaCl: 0.07 mM triethanolamine;

f. fructose 5%.

In each of the systems mentioned solubilizing BPA dissolved to saturation.

The table below shows the results of BPA solubilization study expressed in mg/mL for each of the systems mentioned above.

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Dissolution System	Dissolution degree (mg/mL)
Water	0,1
Water: ethanolamine: HCl 5N	35
Water: triethanolamine: HCl 5N	40
Phosphate buffer with pH = 7.4	0,25
50 mM glucose: 50 mM NaCl: 0.07 mM triethanolamine	40
Fructose 5%.	30

Table 1
BPA SOLUBILITY

It is noted that the degree of dissolution increases in weak bases solutions (mono- and triethanolamine) and solutions of carbohydrates (monosaccharides).

From the systems studied will be selected for future studies those systems with a solubility that meets the conditions for an injectable solution.

Finally, for our experiment, fructose 5% solution was selected.

Studies of bio distribution and BPA capture in animals bearing RS1hepatoma

A highly sensitive method by which we can evaluate the biodistribution and tumor uptake of BPA, respectively the amount of ^{10}B, is the method using as the tracer radio-labeled BPA.

The chemical structure of BPA enables ^{131}I marking by electrophilic substitution mechanism, the electrophilic species I^+, being oriented in the ortho position, as shown in figure 1.

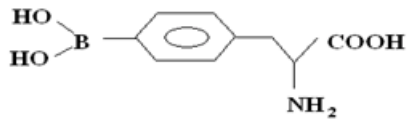

Fig.1. BPA chemical structure

BPA marking with **^{131}I**

To a sample of 10 μg BPA dissolved in 40 μL 0.01 M phosphate buffer, *p*H = 7.4 are added 10 mL chloramine T solution 1 mg / mL.

To this mixture were added 10 μL $Na^{131}I$ solution with 2 mCi activity, and was stirred for 3 min at room temperature.

After the reaction period 5 μL of sodium metabisulphite solution were added in a concentration of 2 mg / mL to quench the reaction and stabilize the final product ^{131}I-BPA.

The radiochemical purity of ^{131}I-BPA determined by radio chromatography on Whatman no. 1 paper, in solvent butanol: acetic acid: water (5: 2: 1 v / v) was 94-96% and the specific activity of the sample was 192 mCi / mg BPA.

Results and disscutions

^{131}I-BPA bio distribution

The model animal used for the studies consists of HRS1 type tumor-bearing Wistar rats, subcutaneous induced, in the top flank of the right foot.

For bio distribution studies and tumoral capture of ^{131}I-BPA, rats were intravenously injected with a volume of 1 mL solution containing 35 mg BPA and an activity 50 μCi ^{131}I-BPA as tracer.

Post injection slaughter times were: 0.5; 1; 3; 5 and 24 h.

There were assigned three rats for each investigation time and interest organs were collected. For each organ, analytical balance weighing and radioactivity measurements (radioactivity measurements were corrected with the disintegration factor according to half-time of ^{131}I - 8.05 days and post injection slaughter time) were performed.

In tabel 2 are presented the results obtained in the ^{131}I-BPA study.

The results indicate a preferential uptake of ^{131}I-BPA at tumoral level with an accumulation maximum at 3 h post injection.

From figure 2 it is observed that capture index defined as the ratio between % Di/ g tumor and % Di / g blood for every investigation time: 0.5; 1; 3; 5 and 24 h is: 1.18; 1.72; 3.46; 2.77 and respectively 1.60 with a maximum value at 3 h after injection.

From the processing of data regarding biodistribution and ^{131}I-BPA capture at tumor level, it is quantitatively estimated the BPA content and respectively ^{10}B captured by tumor (table 2 and fig. 2).

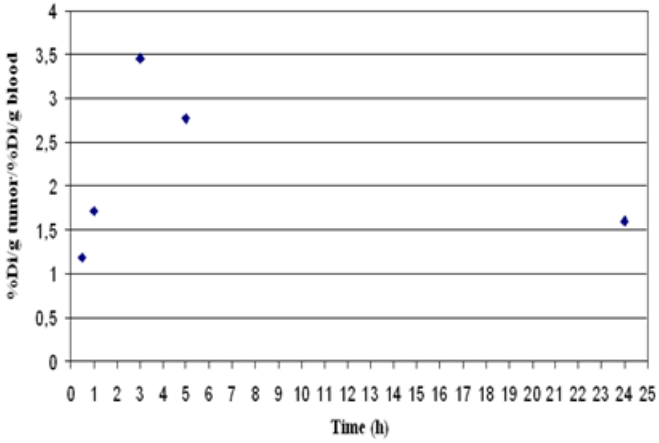

Fig. 2. ^{131}I- BPA capture index in the tumor over time

According to the results shown in table 3, the number of ^{10}B atoms calculated in a gram of tumor tissue (the literature data estimates 10^9 tumor cells / gram of tumor tissue) is 10^{12} - 10^{17} atoms.

In vitro studies for BPA internalization in liver tumor cell HUH-7-17 type

Samples of liver tumor cell HUH-7-17 type were incubated in the presence of variant BPA concentrations.

The experiment stages were:

-Preparation of BPA solutions with different concentrations and ^{131}I-BPA tracer in duplicate.

Organ	Post injection slaughter time (hours)				
	0,5	1	3	5	24
Tumor	3.21	4.15	6.38	5.02	1.62
Blood	2.70	2.41	1.84	1.81	0.98
Liver	2.61	1.85	1.60	1.45	0.81
Brain	0.19	0.14	0.19	0.42	0.28
Muschi	0.61	0.60	0.42	0.62	0.47
Bone	0.25	0.42	0.36	0.21	0.28

Table 2
^{131}I-BPA BIO DISTRIBUTION EXPRESSED in % Di/g organ

Post injection time (hours)	BPA (μg/g tumor)	^{10}B (μg/g tumor)
0,5	1281	60
1	1667	83
3	2554	127
5	2003	100
24	648	32

Table 3
BPA AND ^{10}B AMOUNT AT TUMORAL LEVEL

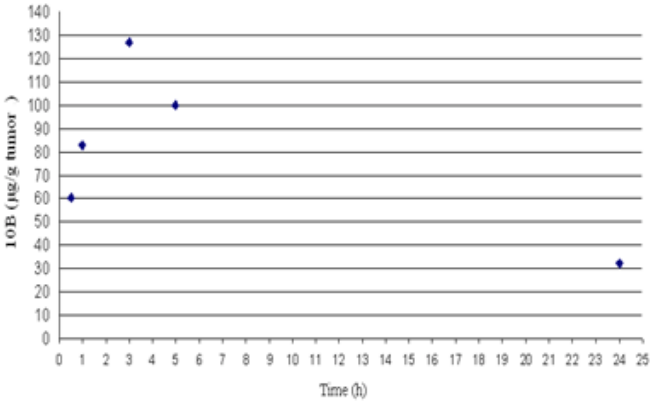

Fig. 3. The variation of ^{10}B/g tumor amount in time

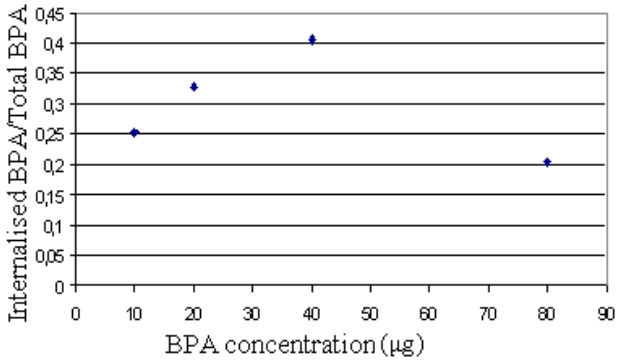

Fig. 4. BPA internalization degree variation

The concentrations of the tested solutions are: 10; 20; 40 and 80μg / mL, each solution receiving an addition of 20 μCi ^{131}I-BPA.

-Incubation parameters are: incubation time 3 h, incubation temperature 37°C under an carbon dioxide atmosphere, the volume ratio 1: 1 between the tumor cell suspension and the BPA solution.

-HUH-7-17 cells (about 5x10^6 cells) samples in duplicate in the presence of ^{131}I-BPA and BPA after incubation were centrifuged for 10 min at a speed of 48.000 rot / min.

-After centrifugation, the supernatant and precipitate were separated, the precipitate was washed twice with 1 mL of water. Radioactivity measurements for each worked samples was done, maintaining constant the volume parameters and measurement factors.

-The values regarding the radioactivity determined at supernatant level and precipitate were processed in order to assess the BPA internalisation degree (table 4).

The BPA internalization degree was defined as the ratio between the BPA amount internalized (measured in the precipitate) and the total amount of BPA present in the sample.

There was a maximum value recorded for BPA internalisation degree in the tumor cell in the 20-40 μg concentration range (fig .4).

For the study of cytotoxic BPA effect BPA solutions samples with different concentrations were delivered in order to assess the correlation between cytotoxic effect and the administration dose of this substance.

Cytotoxic response determination of human lymphocytes cultured *in vitro* under the action of disruptive external agents may be carried out using the test based on determining the micronucleus level (MN).

The micronucleus are formed during the transition from metaphase / anaphase to mitosis (cell division).

MN may occur from an entire chromosome (aneugenic event leading to chromosome loss) or from an acentric chromosome fragment which is detached from a chromosome after breakage (clastogenic event) and do not integrate into the daughter nucleus.

This procedure is applicable for testing the mutagenic effect due to various physical and chemical agents like: radiation, magnetic field exposure in the first division of cells in culture, direct contact with chemical or pharmacological agents.

The test is an *in vitro* method that can also use cell lines outside the primary lymphocytes culture, such as fibroblasts and exfoliated epithelial cells.

The technique ability to determine the MN level, to detect clastogenic and aneugenic effects represents an advantage of this method [7].

Micronucleus frequency analysis in peripheral blood lymphocytes is considered by the IAEA [8] as a bio-indicator of the exposure to ionizing radiation. It is therefore recommended in radiopharmaceuticals genotoxicity effects studies.

In the present study, we aimed to establish the genotoxic effect of boron phenylalanine (BPA) on human lymphocyte cultures.

The experimental parameters characterizing the genotoxicity and cytotoxicity were: the level of micronucleus induced in culture and the index of cell proliferation after incubation with BPA in the 0.5-13.3 mg / mL concentration range.

Micronucleus level determination in in separate lymphocyte cultures

Incubation conditions with BPA: Lymphocytes (in fact the whole population of white blood cells) were isolated by centrifugation in density gradient on Ficoll-Hypaque (Sigma), then washed and re suspended in standard growth medium (RPMI 1640, 10% fetal bovine serum, 2 mM L-glutamine, 25 IU / mL penicillin and 25 IU / mL streptomycin - Sigma).

μg BPA / sample	mg BPA	
	Precipitate	Supernatant
10	2.53	6.67
20	6.53	11.77
40	16.24	18.96
80	16.33	55.97

Tabel 4
BPA INTERNALIZATION PROCESS EVALUATION

BPA concentration in the medium	Experimental parameters		
	Y (MN/1000BN)	*MN induced by treatment*	*CBPI*
0 mg/ml	18.3	0	1.57
0.5 mg/ml (2.5 mM)	20	1.7	
1 mg/ml (5 mM)	21	3	
1.33 mg/ml (6.6mM)	44.4	26.1	1.57
13.5 mg/ml (67.5mM)	71.1	52.8	1.38

Tabel 5
MN LEVEL DEPENDING ON BPA CONCENTRATION IN THE MEDIUM

Lymphocytes isolated from three healthy donors were incubated with various BPA concentrations in the sample: 0.5 mg / mL; 1 mg / mL; 1.33mg / mL; 3.3 mg / mL; 5 mg / mL; 13.5 mg / mL. Incubation was carried out for 2 h at 37°C.

The cell density during the incubation with BPA was ~ 10^6 cel / mL. After incubation, the cells were washed in order to remove unincorporated substance and were turned cultures for micronucleus level determination.

Preparation of cell and culture conditions

For proliferation induction, lymphocytes (treated or not treated with BPA) were suspended in standard growth medium supplemented with 1μL / mL phyto-hemagglutinin (PHA - Sigma - 10 mg / mL).

The lymphocytes in the overall culture (comprising all monocyte subpopulations from peripheral blood) were maintained in plastic culture dishes at a cell density of 0.4 x 10^6 viable cells / mL of medium and incubated in 5% CO2, 80% humidity and at 37 °C. At 39-40 h after stimulation with phytohemagglutinin cytochalasin B (6 μg / mL) was added in order to block cytokinesis.

The samples processing after standing in culture

After 70 h of standing in culture in an incubator at 37°C, the culture was stopped and placed on slides (according to the procedure described above) for the determination of micronucleus (MN) in the lymphocytes.

MN level was expressed by Y parameter defined as the micronucleus number in 1000 binucleate cells from each sample.

Preparations blocked in cytokinesis were also analyzed in order to determine the proliferation index (CBPI), by counting the percentage of cells with 1, 2, 3 and 4 nucleus (N) from a total of 1000 cells.

The CBPI parameter was calculated using the following formula:

CBPI= [(cells no. with 1N) + 2*(cells no. with 2N) +3*(cells no. with 3N and 4N)] / (total cells no.)

The resuls are presented in tabel 5.

The results obtained show that the MN number induced by incubation the lymphocytes cultures with BPA increases with increasing the BPA concentration in the environment, for concentrations in 0.5 - 13.5 mg / mL range.

However, up to 1 mg / mL (equivalent to 5 mM) BPA concentration, this increase is insignificant, being in the inter-individual variability limit for spontaneous MN.

The range of boron concentrations studied (about 25 - 600 ppm) is wider than the concentration range used for boron administration in humans (30 ppm) and other mammals.

Oliveira et al. [9] in the study made for highlighting the cytotoxic effects on melanoma cells (A2058) showed that BPA does not cause genotoxic effects by itself in the 0.5-2.4 mM concentration range.

It is noted that the proliferation index decreases slightly with increasing BPA concentration, but remains within inter-individual variability limits for the proliferation index of control samples.

The results show the cytotoxicity absence for the studied compound, which allows the recommendation of its use in the concentrations up to 1 mg / mL.

Conclusions

After following this first laboratory study regarding BPA use in cancer radiotherapy by neutron capture are assessed the following issues:

- BPA is a substance with a limited degree of solubility in aqueous solvents. Our results allow the selection of conditioned aqueous systems which provides an increased BPA solubility degree, ranging between 30-40μg / mL allowing the saturation with BPA in the tumor cell in order that neutron capture reaction to take place and release α particles with high linear energy transfer, effective in the therapy.

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References

1.BARTH, R.F., CODERRE, J.A., VICENTE, H, Clinical Cancer Research **11,** 2015**,** p.3987-4002
2.SAUERWEIN, W.A.G., WITTIG, A., MOSS, R., NAKAGAWA, Y., Principles and Roots of Neutron Capture Therapy – in Neutron Capture Therapy: Principles and Applications, Springer, 2012, p.1-18
3.HUANG, J., Boron Neutron Capture Therapy for Cancer Treatments - A dissertation submitted to the Department of Physics, University of Surrey, in partial fulfillment of the degree of Master of Radiation and Environmental Protection - Department of Physics, Faculty of Electronics & Physical Sciences, University of Surrey, 2009, p 1-25
4.YOKOYAMA, K, MIYATAKE, S., KAJIMOTO, Y., J Neurooncol., **78**, 2006 , p.227-32
5.SOLOWAY, A.H., TJARKS, W, BARNUM, B.A., Chem Rev., **98**, 1998, p.1515-1562
6.HAWTHORNE, MF, Angew Chem Int Ed Engl, **32**, 1993, p.950–84.
7.FENECH, M., Mutat. Res., **455**, 2000, p.81-95
8.IAEA International Atomic energy Agency Technical Report series, Cytogenetic for radiation dose assessment - a manual, Micronucleus assay, IAEA, Vienna, **405**, 2001, p. 81-89
9.OLIVEIRA, NG., CASTRO, M., Mutagenesis, **16**, 2001, p.369-375

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