Inhibitory Effect of (2R)-1-(1-Benzofuran-2-yl)-N-propylpentan-2-amine on Lung Adenocarcinoma

PhD Theses

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1. Introduction

The incidence of tumors differs between men and women, but lung cancer is highly represented in both sexes and it claims the most life among cancer types. One of the most important etiological factor in lung cancer development is smoking. Other factors such as eating habits, genetic susceptibility and air pollution are also suspected to play relevant roles. Some components of cigarette smoke are metabolized by the cytochrome P450 system, where they form directly carcinogenic agents and bind to the DNA, such as aromatic carbohydrates and nitrosamines. These DNA damages may be repaired or the cell may undergo apoptosis. If none of these happens, the damage in the DNA is fixed and could be inherited by daughter cells. The mutations can occur in genes of key regulator proteins, such as oncogenes and tumor suppressor genes. Kirsten rat sarcoma (KRAS) and epidermal growth factor receptor (EGFR) are examples for the former, while p53, $p21^{CIP1}$ and $p16^{INK4}$ represent the latter. The incidence of KRAS mutation is much higher in lung cancers where patients have a history of smoking.

Based on their phenotype and clinical behavior, lung cancers can be divided to small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), from which the former represents 85% of lung cancer cases. In NSCLC, the dominant subtype is adenocarcinoma characterized by cytokeratin-7 (CK7) and thyroid transcription factor-1 (TTF1) expression. Regarding to their clinical behavior, NSCLC has much better prognosis than that of SCLC, and its therapeutic opportunities greatly increase as well.

Classical chemotherapeutic protocols still make up the major part of NSCLC therapy. The most common use of these cytotoxic agents is in the form of adjuvant therapy, which is used to inhibit the tumor relapse after surgical removal. In this type of tumor, platinum compounds, such as cisplatin, are the most frequently applied agents, where they are combined with other cytotoxic compounds.

Lung adenocarcinoma is one of the first cancers where the role of driver mutations was recognized. The therapeutic options are largely determined by the harbored mutations, which are frequently developed in the genes of EGFR and KRAS. These mutations are usually appear in the so called “hot spot” regions, namely the exon 2 in the case of KRAS, and the exons 18, 19 and 21 of EGFR, coding for the tyrosine-kinase domain. This will result in a mutant protein providing constant self-sustaining growth signal for the cell. EGFR is a transmembrane protein, which binds growth factors and sends a growth signal to the cell nucleus through a complex phosphorylation cascade. The members of this signaling cascade are KRAS-MEK-ERK proteins, which are also connected to other pathways. such as the mTOR pathway. In 15% of the cases EGFR harbors some type of mutation, the incidence of KRAS mutation is around 30%.

The drugs developed against mutant EGFR proteins give most of the targeted therapies available for lung adenocarcinoma. Erlotinib, gefitinib and lapatinib were the first tyrosine-kinase inhibitors (TKI). Activating mutations in the KRAS gene are the most frequent alterations in the signaling pathway of lung adenocarcinoma. In this case, no therapeutic benefit can be expected from EGFR inhibitors, which makes KRAS mutation analysis one of the first step in the diagnostic process. Up to date, there is no targeted therapy available for the mutations of KRAS.

The deeper knowledge of the molecular background of cancers, and the appearance of new targets enable us to broaden the therapies applied. However, already available medicines used in other indications may be rediscovered as anti-tumor agents.

The phenylethylamine derivate (-)-deprenyl was the first drug inhibiting the MAO-B enzyme and it became a key substance in the therapy of Parkinson’s disease. It was concluded from longevity studies that DEP can prolong life span in mice, rats and beagle dogs after daily treatment with the MAO-B inhibitory dose (0.25 mg/kg). These findings resulted in the development of the tryptamine derivate BPAP. BPAP also has an enhancer activity on the adrenergic and serotonergic neurons in the central nervous system, but it has no effect on the MAO-B enzyme. Both

molecules exert neuroprotective and memory stimulating effects. BPAP can provoke these effects in a very low concentration range.

Based on these results, the effects of DEP and BPAP were examined in additional longevity studies carried out in Wistar rats, where the life prolonging effect was proven again in this low concentration range. Beside the memory stimulatory and the life prolonging effects, the decreased incidence of strain-specific spontaneous fibrosarcoma was also noted in the DEP and BPAP treated groups. Administration of the two compounds resulted in delayed tumor appearance and reduced tumor number compared to control group. BPAP was more effective in tumor inhibition.

Based on these observations, BPAP was tested in other tumor models. In an *in vivo* model of colon cancer with liver metastasis, BPAP significantly reduced the number of metastasis. The effect of BPAP is multifaceted: it is capable of prolonging lifespan, stimulate learning and affect behavior in a very low concentration. In our work, we present the antitumor effect of BPAP in lung adenocarcinoma and we map the underlying mechanism of action.

2. Aims

- To characterize the DEN-induced lung cancer model, to search for the most common mutations of lung adenocarcinoma and to evaluate the activity of signaling pathways.
- To examine the inhibitory effects of BPAP in our mouse lung cancer model and to evaluate its effects on other human lung adenocarcinoma cell line xenograft model in SCID mice.
- To map the possible mechanism of action.

3. Methods

***In vitro* experiments**: Human lung adenocarcinoma cell line (H358) was applied. The direct effects of BPAP were evaluated by sulphorhodamine-B proliferation assay. This cell line was also utilized for a xenograft model.

***In vivo* experiments:** Lung cancer was induced in FVB/N mice strain with a single dose of diethyl nitrosamine (DEN). From these lung tumors, subcutaneously maintained allograft tumor cell line was created. In parallel, spontaneous tumorigenesis in control untreated animals was also monitored. Two experiments were carried out on the subcutaneous allograft lung adenocarcinoma model to evaluate the effects of BPAP. The experiment was repeated the 3rd time in SCID strain with the same protocol. The effect of BPAP was also investigated in a H358 xenograft model. In all experiments one group was treated with 0.0001 mg/kg (low dose) and another with 0.05 mg/kg (high dose) BPAP, while the control group received saline.

The tissue samples harvested from these experiments were used for histological/immunhistochemical analyses and molecular evaluations.

Mutation analysis: All the tumor samples induced by DEN were analyzed by Sanger sequencing for mutations in the exons 19 and 21 of EGFR and in the exon 2 of KRAS. The analysis was conducted on the two spontaneously developed lung tumors and also on the subcutan allograft tumor.

Protein activity and diagnostic evaluation: The diagnostic adenocarcinoma markers TTF1 and CK7 were used to characterize the tumors by immunohistochemical procedure. Phospho-receptor tyrosine kinase activity was measured by phospho-RTK Proteome Profiler Array. The activity of signaling pathways in the different groups were compared by **Western blot**. The following protein levels were measured: Akt, p-Akt (Ser473), p-Akt (Thr308), p-Erk 1 and 2, p-S6, CDK4, PCNA, Cyclin D, p-Rb (S780), $p16^{INK4}$, NF-κB, c-jun and β-actin.

All **statistical analyses** were performed using GraphPad Prism 7.00 software (Graphpad Software Inc., La Jolla, CA, USA). Data were tested for normal distribution using the omnibus normality test of D'Agostino & Pearson. Significance of changes were tested using a nonparametric test (the Mann– Whitney U-test) or the Student's t test,

depending on the distribution of the data. The independent experimental sets were compared for reproducibility. Only reproducible significant changes were considered as significant. Significance was declared at the standard $p < 0.05$ level.

The Western blot results were calculated by Microsoft Office Excel. The results of the animal experiments are showed with standard error, and the results of the Western blot are presented with standard deviation.

4. Results

The tumor inducing potential of DEN and characterization of tumors

After termination, macroscopic tumors were found in 28 animals out of 39, where 6 mice had tumors with multiple foci. The tumor incidence did not show any correlation with the gender. In the control group only 2 mice developed spontaneous tumors out of 14.

High expression of TTF1 and CK7 proteins was found in each sample, confirming the adenocarcinoma type. The positivity was also observed in the spontaneous tumors.

Each tumor sample was tested for mutations in the exon 2 of KRAS, and in exons 19 and 21 of EGFR. All of them was found to be wild type.

Next, we compared the activity of Akt/mTOR and MAPK signaling pathways in normal lung tissue, DEN-induced primary and subcutan tumors. The p-Akt Thr308 decreased 40% in the tumors compared to the normal tissue, while the p-Akt Ser473 decreased in the subcutaneously maintained tumor and showed a slight elevation in the primary tumor.

The p-Erk 1 and 2 levels were raised in the subcutan tumor with 20% and 80% compared to healthy lung. No change was seen in the primary tumor. The level p-S6 increased with 5-fold in both groups compared to the normal lung tissue.

We analyzed the changes in the cell cycle where a marked increase in the level of CDK4 was found at the G1/S state. The amount of CDK4 elevated with 4-fold in the primary tumor and tripled in the subcutaneous tumor compared to the normal tissue. PCNA showed an 18-fold increase in the primary tumor and 24-fold increase in the subcutaneous tumor. The level of p-Rb elevated in the primary tumor, but its level did not change in the subcutaneous tumor.

The effects of BPAP on the mouse lung cancer

Results of animal studies

The tumor inhibitory effects of BPAP were monitored in two independent experiments, where both doses of BPAP significantly

decreased tumor growth. Tumor volumes were reduced by 40% in both studies by the small and high doses of BPAP. In addition, BPAP effectively attenuated the symptoms of cachexia, and resulted in delayed cancer-related wasting. From these results we concluded that BPAP may inhibit the development of cachexia, where high dose seems to be more effective in maintaining body weight.

The action of BPAP was also tested in SCID mouse strain to decide whether the adaptive immune system plays a role in exerting the effects of BPAP. Similarly to the results of the previous experiments in the FVB/N strain, BPAP significantly inhibited tumor growth in this immunocompromised strain. The high dose showed to be less effective, but it still decreased tumor volume by 30%. By the end of the study the tumors in the low dose treated groups were smaller by 70% than that of the control group.

Western blot results

The effects of BPAP were examined on the two main signaling pathways of lung adenocarcinoma namely mTOR/Akt and MAPK on tumor samples of SCID allograft model by Western blot analysis. Our results revealed that the two doses of BPAP acted differently in our tumor model.

The low dose doubled the amount of both p-Akt Ser473 and Thr308, while the high dose decreased the p-Akt Ser473 level by 50%, and the p-Akt Thr 308 by 75%. Similar changes were noted in the case of p-Erk 1 and 2. The low dose treatment elevated them by 40% and 50%, while the high dose treatment reduced them by the same amount. The level of p-S6 was raised by both doses, a mild 30% increase was provoked by the high dose, and a marked 90% elevation by the low dose. NF-κB was decreased by 10% after the low dose, and by 40% after the high dose of BPAP, respectively. We detected a 15% increment in the level of c-jun after low dose treatment, but it decreased by 30% when high dose was applied.

When analyzing cell cycle progression, significant changes were detected in the level of regulator proteins at the G1-S checkpoint provoked by BPAP treatment. The level of p-Rb Ser780 decreased by 50% in both treated groups. Cyclin D1 amount also dropped by 40% after both high and low dose exposure. CDK4 level was reduced to 50%

by the low dose, and a 20% decrease was also measured after high dose treatment. The S phase marker PCNA decreased by 35% upon exposure to low dose, and by 10% in the high dose group. The level of $p16^{INK4}$ doubled in samples treated with low dose BPAP, while it showed no significant change after high dose treatment.

The results of cell cycle regulatory proteins point to that both BPAP doses inhibit proliferation of tumor cells by provoking cell cycle arrest at G1-S checkpoint despite their different acts on signaling pathways.

p-RTK assay

Tumor samples of SCID mice treated with BPAP doses or saline were applied on a phospho-RTK assay. Phospho-PDGF-Rα levels were quantified by densitometrical analysis of dots. Mean of data were calculated and control, low dose and high dose of BPAP treated groups were compared. There was no significant difference in the level of receptor after either dose of BPAP vs control.

The effects of BPAP on H358 lung adenocarcinoma cell line

In vitro results

BPAP did not affect the proliferation rate of H358 human lung adenocarcinoma cell line tested by SRB cell proliferation assay. As we have no information of the exact concentration of BPAP in the plasma after treatment, BPAP's effects were tested in a given concentration range. Although treatment doses were only approximate to the actual doses applied in the *in vivo* studies, it is likely that some inhibitory effect would have been detected if BPAP directly acts on tumor cells.

Xenograft model

H358 tumor cells were implanted subcutaneously in SCID mice and treated with low and high dose of BPAP. No effect of low dose treatment was observed until the 40^{th} day of the experiment, but after that a slight tumor inhibition was noted. By the end of the study a nearly 30% decrease in tumor volumes was measured compared to the control group. The high dose of BPAP showed a much more significant effect

throughout the whole study. At termination these tumors were found to be 65% smaller than that of the control animals.

Western blot

The analysis of signaling pathways in the BPAP treated H358 cell line did not lead to results similar to those obtained in the FVB/N mouse lung cancer model. There was no change in the level of p-Erk1/2 or in p-S6. The low dose elevated both p-Akt levels. The amount of Ser473 variant doubled, but it showed no significance due to high deviation, while the Thr308 variation increased by 40%. The high dose did not have any effect on either phospho-Akt level. Interestingly, the low dose treatment elevated the level of CDK4 by 30%, while the high dose had no effect in this case. High dose BPAP reduced the amount of PCNA by 35%, but low dose had no effect on it. High dose also reduced the amount of p-Rb by 35% similarly to PCNA, but the low dose showed no change here either.

5. Conclusions

The characterization of the lung adenocarcinoma model of FVB/N strain

DEN is a well-known and often used substance to induce liver cancer in animal studies. It acts as a strong alkylating agent which forms adduct with the DNA after its bio activation by the cytochrome P450 system, which will later result in a direct carcinogen effect. In some cases it was shown that nitrosoamines can also induce lung cancer in such strains where the incidence of spontaneous lung cancers are already higher than other cancers.

The lung cancer induced in FVB/N strain showed strong positivity of CK7 and TTF1 markers by immunhistochemistry, and the hematoxylin-eosin staining also strengthened the diagnosis of adenocarcinoma. None of the tumors had mutation in either the sequenced EGFR exons or in the KRAS exon. The susceptibility of FVB/N strain for lung cancers is significantly higher than for any other cancer type, while the incidence of liver cancer is quite rare in them. DEN could have enhanced the already high susceptibility for lung cancer, accelerating the tumorigenesis. This DEN induced lung adenocarcinoma model has great significance as lung cancer is one of the most frequently examined tumor type and it can serve as a good model for further studies aiming therapy.

We started the characterization of signaling pathways with the mTOR network, because it has a central role in the life of the cell and it connects with the EGFR/KRAS pathway, a key signaling in lung cancers. After activation, mTOR has multiple effect in the cell. It enhances cell growth, protein synthesis, cell cycle progression. It affects cell survival, metabolism, lipid synthesis and the cytoskeleton formation. Its overall effects are cell survival and progression. The DEN induced tumors displayed high p-S6 levels, reflecting on the mTOR activity. The constant stimuli of proliferation by mTOR causes a fully active cell cycle. These changes can be seen in the primary and subcutan tumors. The subcutaneously maintained tumor has higher cell cycle activity which confirms the more aggressive phenotype on the molecular level, compared to the primary tumor.

The effects of BPAP in vivo on the lung adenocarcinoma model in FVB/N strain

BPAP effectively inhibited tumor growth in the FVB/N strain. The overall results are basically identical when the effects of two treatment doses are compared, but they completely differ in their acts on the signaling pathways. Inverse effects of the low and high dose BPAP were detected on p-Akt Ser4703/Thr308 and p-Erk 1 and 2 levels. The high dose exerted a direct inhibitory effect on the level of these signaling proteins, while the low dose significantly increased their activity. Based on these, low dose BPAP treatment should have led to an acceleration of proliferation rate. On the contrary, analysis of the cell cycle proteins indicated that there is a strong G1-S phase block after both treatments, which correlates with the smaller tumor volume after low and high doses of BPAP.

The level of c-jun was significantly altered by both doses. C-jun is an oncogene and its level was reduced by high dose of BPAP, which fits to the concept of the overall effects. Interestingly, the low dose increased c-jun level in parallel with the active form of Akt and Erk. Based on these results, we concluded that the two doses of BPAP may act with a completely different mechanism. The opposing effects of low and high dose of BPAP could be explained by the phenomenon of geroconversion.

Geroconversion is a form of cell growth, which occurs during cell cycle block, when despite of the high level of growth signals, the cell can not proliferate. Geroconversion is caused mostly by high mTOR activity. The $p21^{CIP1}$ and $p16^{INK4}$ tumor suppressors stop division of hyperproliferating cells, but even under cell cycle blockade, growth signals may still be present. As cell division is arrested by tumor suppressors, growth signals lead to a cell morphology of senescence and cells lose their ability to restart their proliferation. This phenomenon is called geroconversion. Our results showed that the low dose BPAP treatment caused a high level of mTOR activity, but there is also a G1 phase block present and the amount of p16 is doubled compared to that of high dose and control group. There was no sign of geroconversion in tumors treated with high dose BPAP.

Both treatments decreased the level of NF-κB, but the high dose exerted a much stronger effect. NF-κB plays a key role in the development of cachexia. Cachexia is a condition of end-stage cancer patients, a complex disease that indicates the last stage of the disease and leads to the death of the patient. During the experiments on FVB/N strain, animals treated with BPAP had less weight loss. By the end of the study, the average bodyweight was higher by 14% and 24% in low and high dose groups respectively. These differences in the bodyweights correlate well with the level of NF-κB measured in the tumor samples.

Our experiments in SCID strain aimed to determine whether the adaptive immune system has any role in the mechanism of effect of BPAP. This strain has no adaptive immune system, thus serving as a good model for xenograft experiments. The low dose BPAP treatment resulted in a similar tumor inhibition as seen in the FVB/N strain. The high dose treatment led to a weaker, but still convincing growth inhibition. Based on these results we concluded that the adaptive immune system has no significant role in the mechanism of action of BPAP.

The effects of BPAP on H358 human lung adenocarcinoma cell line in in vitro and in xenograft model

Unfortunately, we failed to start primary cell cultures from the lung tumors induced in FVB/N strain. This would have been a helpful model to clarify whether BPAP exerts direct effects on tumor cells.

Alternatively, we tested BPAP action on the H358 human lung adenocarcinoma cell line under *in vitro* conditions, but no inhibition was noted. This by good chance means that BPAP does not have a direct effect on tumor cells, but to decide, we tested this cell line *in vivo* in SCID strain, as well.

H358 tumor cells were implanted subcutaneously in SCID mice. In this case, BPAP treatment led to inhibited tumor growth. A relatively weak, non-significant result was seen in the low dose group, but a very strong, 65% tumor volume reduction was observed upon high dose treatment. The H358 cell line is a human lung adenocarcinoma line, which has a confirmed G12C KRAS mutation. This mutation is often described in smoking patients. Based on these results we assume that

BPAP may be a treatment option in the case of KRAS mutation, but the mechanism behind the action remains unclear.

From our results the following conclusions can be drawn:

1. A single small dose of DEN can induce lung tumors in FVB/N strain, which has a higher susceptibility for lung cancer due to its genetic background.
2. All DEN induced and the spontaneous tumors showed adenocarcinoma histological type, and neither of them had mutation in the EGFR or KRAS genes.
3. The tumor has high mTOR activity.
4. BPAP effectively inhibited the growth of subcutaneous tumors both in FVB/N and in SCID strains.
5. The effects of BPAP is independent of the adaptive immune system.
6. BPAP effectively inhibited the weight loss caused by cachexia in a dose dependent manner.
7. The two doses of BPAP acted differently at the molecular level. The high dose exerted a direct inhibitory effect on the signaling pathways, while the low dose showed the signs of geroconversion.
8. BPAP had no effect on H358 lung adenocarcinoma cell line *in vitro*, but it had a strong inhibitory effect on tumor growth *in vivo* in SCID mice indicating an indirect mechanism of action.
9. BPAP high dose strongly inhibited growth of H358 tumor cells even though it harbors a KRAS mutation.

6. Publications

Publications in context of the thesis:

1. Mervai Z, Egedi K, Kovalszky I, Baghy K. (2018) Diethylnitrosamine induces lung adenocarcinoma in FVB/N mouse. BMC Cancer. 18: 157.

2. Mervai Z, Reszegi A, Miklya I, Knoll J, Schaff Z, Kovalszky I, Baghy K. (2019) Inhibitory Effect of (2R)-1-(1-Benzofuran-2-yl)-N-propylpentan-2-amine on Lung Adenocarcinoma. Pathol Oncol Res.

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