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# The Effects of Isopropyl Methylphosphono-Fluoridate (IMPF) Poisoning on Tumor Growth and Angiogenesis in BALB/C Mice

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Data Interpretation D  
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**Background:** Acetylcholinesterase (AChE) and cholinergic receptors have an important role in the immune system and angiogenesis. This work evaluated the effects of isopropyl methylphosphonofluoridate (IMPF), an irreversible inhibitor of AChE, on tumor growth and selected parameters associated with tumor angiogenesis.


**Material/Methods:** Experiments were performed on male BALB/c mice exposed to IMPF (study group) or saline buffer (control group) and inoculated with L-1 sarcoma; the number of new blood vessels (TIA test) and the level of  $\alpha v \beta 3$  integrin ( $^{131}I$ -MAB-anti $\beta 3$  assay) were analyzed at seven, 14, or 21 days after implantation of the tumor cells.

**Results:** The IMPF poisoning affected tumor angiogenesis (TIA test). There was a statistically significant increase in the number of newly forming blood vessels in the group subjected to IMPF and inoculated with tumor cells.

**Conclusions:** This study showed that IMPF had a significant effect on the regulation of lymphocyte-induced angiogenesis and the modulation of angiogenic and pro-inflammatory cytokines secretion. The observed effects suggest involvement of neuronal and/or non-neuronal cholinergic signaling pathway.


**MeSH Keywords:** **Angiogenesis Inducing Agents • Cholinesterase Inhibitors • Sarin**

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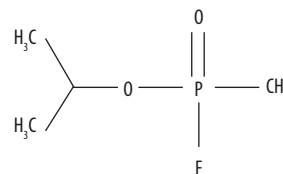
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## Background

Isopropyl methylphosphonofluoridate (IMPF, also called “sarin”) is one of the main representatives of the phosphor-organic compounds. IMPF was developed in 1938 by German scientists as a pesticide [1]. One year after its discovery, the development of IMPF was transferred to chemical warfare section of the German Army Weapons as a weapon of mass destruction [1]. Sarin was used several times during military conflicts and against civilian population (in 1988 Iran-Iraq War and on Kurdic civilians from city of Halabja) [2,3]. IMPF has been also used in terrorist attacks in Matsumoto city (1994) and the Tokyo subway system (1995) and caused 12 deaths and about 5,500 injuries [4]. Production and stockpiling of sarin was outlawed in April 1997 by the Chemical Weapons Convention of 1993, however, some countries still have an arsenal of this weapon [5,6]. The last mass use of IMPF was in Syria in April 2017 [6]. The destructive role of IMPF is related to its irreversible inhibition of acetylcholinesterase, which is the main transmitter of the cholinergic system. This enzyme is also associated with angiogenesis (the processes of new blood vessels formation) [7–9]. In normal tissue, angiogenesis is a self-monitoring and self-limiting process. In pathologically changed tissue, there is an imbalance between the activities of pro- and anti-angiogenic factors [10]. Illnesses involving changes in angiogenesis include vascular disease (e.g., hemangioma, angiofibromas), eye conditions (e.g., proliferative retinopathy, glaucoma vascular, trachoma), and diseases of the joints (e.g., rheumatoid arthritis, articular changes in hemophilia), skin diseases (e.g., psoriasis, scleroderma) and cancers [11]. Angiogenesis is also very important in the transplantation process [12,13]. It allows maintaining the transplanted organ and retaining its functionality. However, very little is known of the mechanisms involved in the revascularization of transplanted organ [14].

The highly toxic compound organophosphates may cause alterations in the processes of blood vessel formation. The mechanisms underlying the effects of poisoning must be identified to select appropriate prevention measures and treatment following exposure incidents. Our previous study showed that IMPF affects Th1/Th2 cytokines and antibody concentrations, which may switch the immune response to its humoral pathway in BALB/c mice [15]. Furthermore, IMPF exposure modulates angiogenic cytokines concentration (VEGF, bFGF, TNF- $\alpha$ , and IFN- $\gamma$ ), which decreases the potential of lymphoid spleen cells to induce local graft-versus-host reaction [16]. Based on these results, we hypothesized that a blockage of acetylcholinesterase by IMPF may induce tumor angiogenesis and growth. This knowledge is especially important in the context of long-term care for patients exposed to IMPF poisoning.



**Figure 1.** Isopropyl methylphosphonofluoridate (IMPF).

## Material and Methods

Isopropyl methylphosphonofluoridate (IMPF) (Figure 1) was obtained from the Military Institute of Chemistry and Radiometry in Rembertów, Poland.

### Animals

The study was performed on male BALB/c mice (BALB/CanNCrCmd) obtained from the Institute of Experimental and Clinical Medicine at the Polish Academy of Sciences in Warsaw. The animals were kept at standard conditions (at room temperature 22.5–23.0°C, relative humidity 50–70%, 12-hour day/night cycle) with free access to food and water. Both environmental conditions and animal health were under daily veterinary supervision. The study was approved by the Local Ethics Committee (Decision No. 17/04).

### IMPF poisoning

The animals were divided into two study groups: the study group (received a 0.5 LD<sub>50</sub> dose of IMPF-100 mg/kg body weight in saline solution) and the control group received respective doses of saline. Both saline and IMPF were administered intraperitoneally under anesthesia (0.1 mL of 3.6% of chloral hydrate solution). Immediately after IMPF or saline administration, tumor cells were implanted.

### Tumor cells

Mouse sarcoma L1 cell line (sarcoma) was obtained from the Maria Skłodowska-Curie Institute of Oncology in Warsaw. The cell line was derived from a lung metastasis of a BALB/c mice. We used cells from the seventh and eighth passages, established as a stable growing line (L1) and grafted (10<sup>6</sup> cells/0.1 mL) subcutaneously into each side of the mouse thorax. After 14 days the mice were euthanized, tumors were excised, cut up into small fragments, pressed through a sterile nylon strainer (40  $\mu$ m) and suspended in 5 mL of PBS. After sedimentation (10 minutes) the supernatant was collected and centrifuged (10 minutes, 300 $\times$ g). The cell pellet was washed with PBS, centrifuged and resuspended in Parker medium at a concentration of 4 $\times$ 10<sup>6</sup> of cells/mL. Then 200,000 cells (50  $\mu$ L of obtained suspension) were used for the injection.

### Tumor-induced angiogenesis (TIA) test

The tumor-induced angiogenesis (TIA) test was performed as described by Zdanowski et al., (2012) [17]. Tumor cells were injected intradermally into partly shaved Balb/c mice. Then a seven, 14, or 21 days later the mice were euthanized (pentobarbital (400 mg/kg body mass) Polypharm SA, Poland). All newly formed blood vessels on the inner skin surface were identified and counted under dissection microscope (magnification 6x, in 1/3 central area of microscopic field).

### <sup>131</sup>I-MAb-antiβ3 integrin identification

The assay was performed as previously described by Bilski et al. (2004) [18] using radioactive antibody conjugate (<sup>131</sup>I-MAb-antiβ3) against the β3 integrin subunit αvβ3 (anti-CD61). At 24 hours before autopsy 150 μL of conjugated <sup>131</sup>I and anti-β3 integrin subunit αvβ3 solution was administered to the tail vein.

After that animals were euthanized (pentobarbital, 400 mg/kg body mass), tumors were isolated and washed with cold PBS, dried, weighed, and their radioactivity was measured by single-channel analyzer γ radiation (10 second). The percentage of radioactivity per gram of the tumor mass (%ID<sub>g</sub>) was calculated from the formula:

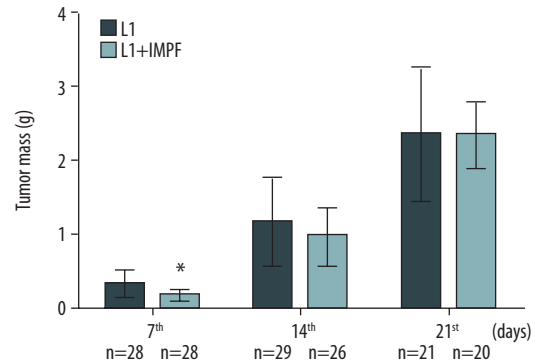
$$\%ID_g = \frac{A_g}{M \cdot \sum A(t)} \cdot 100$$

The tumor uptake values were expressed as the percent of injected dose per gram of the tumor mass (%ID/g).

Additionally, the radioisotope accumulation in healthy tissue was measured. Samples were collected from the femoral biceps (%ID<sub>m</sub>). In both tumor/and non-tumor tissue the antibody accumulation was evaluated as T/NT ratio: T/NT ratio=%ID<sub>g</sub>/<sub>%ID<sub>m</sub></sub>; %ID<sub>g</sub> – the percentage of accumulated radioisotope per tumor mass unit (g);%ID<sub>m</sub> – the percentage of accumulated radioisotope per muscle tissue mass unit (g).

### Statistics

All results were presented as mean ± standard errors (SEM). The characteristics of the data distribution were assessed using Shapiro-Wilk test. In cases of normal distribution, the t-test was used, in other cases we used Mann-Whitney U test. The level of significance was set at *p*<0.05.



**Figure 2.** Average mass ±SD of isolated tumors on selected days; \* statistically significant differences between groups, *p*<0.05; n, the number of animals used in experiments.

## Results

### Tumor mass

Administering isopropyl methylphosphonofluoridate affected the weight of developing tumors in the early stages of growth. Statistically significant reduction in the mean tumor mass was observed on the seventh day after L1 cell inoculation (*p*<0.05). On the fourteenth day the difference was slightly reduced in the IMPF group (not significant) and at the end of the experiment the mean weight of tumors from both groups was almost the same. The results are presented in Figure 2.

### Tumor-induced angiogenesis

IMPF poisoning significantly enhanced the number of newly formed blood vessels; the sarcoma cells administration caused time-dependent increase of the average number of tumor formations on the seventh (over 55%, *p*<0.001) and twenty-first day of experiment (about 35%, *p*<0.001) (Table 1).

We did not observe significant differences between the control and IMPF groups on the fourteenth day of the experiment, which may be related to the high variation of new blood vessel numbers in the IMPF group.

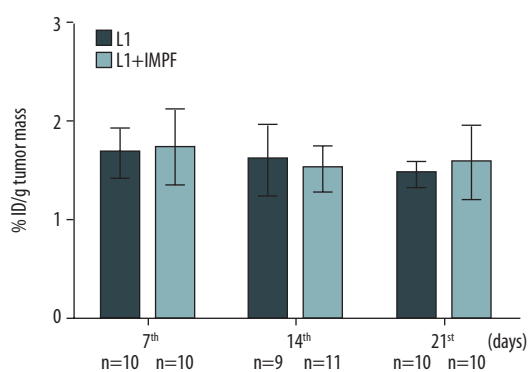
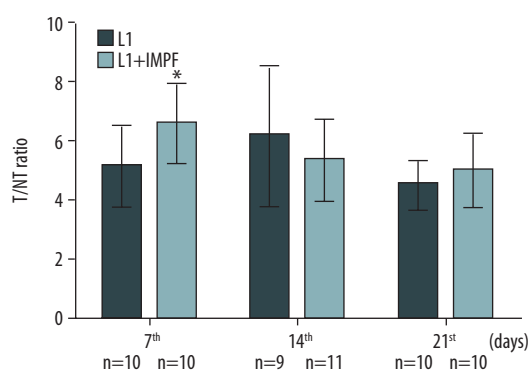
### <sup>131</sup>I-MAb-antiβ3 integrin identification

Tumor angiogenesis, induced by L1 sarcoma inoculation in BALB/c mice, was investigated by a radioimmunoassay technique (anti-CD61 antibody conjugated with <sup>131</sup>I). The percentages of radioisotope accumulation in the tumor mass unit (%ID<sub>g</sub>) on the seventh, fourteenth, and twenty-first day after L1 implantation were similar. There were no statistically significant differences between the control and study groups in

**Table 1.** The average number  $\pm$ SD of newly formed blood vessels in TIA assay.

	L1 $\pm$ SD	L1+IMPF $\pm$ SD	<i>p</i>
Day 7	14.64 $\pm$ 2.307 n=16	23.06 $\pm$ 5.272 n=9	<b><i>p</i>&lt;0.001</b>
Day 14	19.00 $\pm$ 2.739 n=6	20.60 $\pm$ 3.460 n=9	n.s.
Day 21	19.24 $\pm$ 2.818 n=6	25.40 $\pm$ 2.716 n=8	<b><i>p</i>&lt;0.001</b>

*p* – level of significance; bold font – statistically significant differences; n.s. – statistically insignificant difference; n – number of TIA tests.

**Figure 3.** The percentage of isotope accumulated in the tumor mass unit (%ID<sub>g</sub>). n – number of animals used in experiments.**Figure 4.** Accumulated isotope ratio. T/NT, tumor/non-tumor; \* statistically significant differences between groups, *p*<0.05; n – number of tested animals.**Table 2.** Percentage of accumulated isotope per unit mass of muscle Tissue%IDm/g  $\pm$  standard deviation.

	M <sub>L1</sub> $\pm$ SD	M <sub>L1+IMPF</sub> $\pm$ SD	<i>p</i>
Day 7	0.3364 $\pm$ 0.0574 n=10	0.2821 $\pm$ 0.0300 n=10	<b><i>p</i>&lt;0.05</b>
Day 14	0.2417 $\pm$ 0.0627 n=9	0.2956 $\pm$ 0.0791 n=11	n.s.
Day 21	0.3214 $\pm$ 0.0493 n=10	0.3152 $\pm$ 0.0501 n=10	n.s.

ML1 – muscle tissue taken from animals inoculated with L1; ML1+IMPF – muscle tissue taken from animals inoculated with L1 and given IMPF; *p* – level of significance; bold font – statistically significant differences; n.s. – statistically insignificant difference; n – number of animals tested.

radioisotope accumulation after seven, 14, and 21 days of the experiment (Figure 3).

The study also looked at radioisotope accumulation in healthy tissue (femoral biceps). Analyses showed the radioisotope accumulation in healthy tissue was 4–6 times lower than in tumor tissue. IMPF poisoning led to a significant decrease of femoral biceps radioisotope accumulation on the seventh day

of experiment (about 15%, *p*<0.05). There were no significant changes after 14 and 21 days of the experiment. The results are presented in Table 2.

There were no significant differences in T/NT radiological index between the results obtained on the seventh, fourteenth, and twenty-first days of the experiment in the control group. IMPF poisoning caused significant augmentation of T/NT index

only on the seventh day of experiment. Observed changes were mainly the result of a drop in antibody accumulation in the reference tissue (Figure 4).

## Discussion

IMPF poisoning significantly reduces the activity of AChE in certain areas of the brain such as the hippocampus [19]. The hippocampus and hypothalamus are the two main regions of the brain involved in the process of neuroimmunomodulation [20,21]. Disorders of the nervous system may lead directly or indirectly to the modification of certain immunological parameters [22]; the nervous system also controls angiogenesis. IMPF poisoning inhibits the activity of AChE in the brain regions necessary for neuro-immune interactions. It should be emphasized that these processes mainly involve the autonomic nervous system, whose essential components are cholinergic neurons (the parasympathetic nervous system).

In this study, we evaluated the effects of IMPF administration on the dynamics of tumor angiogenesis. We observed a statistically significant increase of tumor neovascularization on the seventh and twenty-first days after intoxication with IMPF administration in comparison to the control group (Table 1). The differences observed may be associated with the kinetics of tumor development. Several days after implantation, a growing tumor reaches the size requiring intensive angiogenesis; which in turn causes an increase of the secretion of pro-angiogenic cytokines by tumor cells or tumor-infiltrating immunocompetent cells [23]. Well-nourished tumor cells start to proliferate faster, perpetuating genetic changes and leading to a metastatic phenotype. Paradoxically cancer angiogenesis is not fully correct. The main role played in tumor mass is hypoxia and subsequent low pH, which causes an abnormal tumor vasculature. It also attenuates immune system response by inducing immune tolerance thought to inhibit T CD8+ cells and NK cells activity [24,25]. An increased level of newly formed vessels, especially at the early stage of our experiment, may have caused an increased concentration of oxygen in the tumor mass and better infiltration of immune cells. This effect could also be enhanced by acetylcholine stimulation (not decompose by AChE inhibition), which dilates the blood and lowers the flow of blood, thereby facilitating gas exchange [26]. This could partly explain the lack of differences in the later period of experiment (at 14 days and 21 days). The increased activity of AChE caused restricted access to oxygen and restore "normal tumor microenvironment".

There is another possible mechanism which IMPF could affect tumor growth. It is well known that acetylcholine plays an important role in immune response (via non-neuronal cholinergic system) [27]. Various immune competent cells exhibit expression

of the ACh receptor. ACh is also released after antigen stimulation of CD4+ T cells [28]. Activation of several components of the immune system by ACh may delay the growth L1 sarcoma in mice observed at the seventh day of the experiment.

Tumor growth requires more nutrients, which further stimulate angiogenesis. The main factors participating in angiogenesis are pro-angiogenic cytokines such as VEGF and bFGF. This is probably one of the mechanisms by which IMPF affects endothelial cells proliferation and migration. As we previously reported, both cytokines were affected by IMPF poisoning in mice [16]. We showed here that a time-dependent change in VEGF and bFGF concentrations after poisoning were associated with an increase in the number of newly formed blood vessels, particularly on the seventh day of the experiment.

The cytokines are not the only molecules affecting the angiogenesis process. Equally important are cell adhesion receptors and glycoproteins, with integrins being particularly significant [29]. The importance of glycoproteins is related to the processes of cell adhesion. It has been shown that integrins may modulate migration, development, and death of endothelial cells [30]. The first report associating glycoproteins with the processes of angiogenesis emerged in 1994, when Brooks et al. observed that newly formed blood vessels exhibited strong expression of integrin  $\alpha v \beta 3$ . Interestingly, the integrin  $\alpha v \beta 3$  antagonist inhibited angiogenesis *in vivo* and induced apoptosis in endothelial cells.

Presently it is known that the expression of  $\alpha v \beta 3$  integrins occurs mainly in the active endothelium involved in the process of blood vessel formation [31].

In this study we used  $^{131}\text{I}$  conjugated antibody against the subunit  $\alpha v \beta 3$  of CD61 as one of the markers of angiogenesis. We have not observed any pronounced changes in the accumulation of antibodies (Figure 3). The absence of significant differences may be a result of individual changes in tumor growth and angiogenesis as well as homogeneity of the tumor. To minimize this effect the percentage of radioisotope accumulation in the tumor mass was compared to the accumulation in the tissue muscle (T/NT ratio) (Figure 4). We observed statistically significant increase of T/NT ratio on the seventh day of the experiment. This was primarily a result of a significant decrease in the expression of  $\alpha v \beta 3$  integrin in the background signal (muscle) after IMPF treatment (Table 2). The absence of differences in radioisotope assay between the control and study groups on the fourteenth and twenty-first days may result from the fact that we only used the antibody against a subunit 3 integrin. The  $\alpha v \beta 3$  subunit is one of the many other integrins jointly modulating the neo-vascularization process. Moreover, various types of integrins may be expressed in vascular endothelium cells depending on the stage of tumor development [32].



It is known that antagonists of integrins  $\alpha\beta3$  and  $\alpha\beta5$  inhibit angiogenesis; however, the way they act is different [33,34]. The  $\alpha\beta3$  inhibitors block angiogenesis through interference with bFGF signaling [35,36], while  $\alpha\beta5$  inhibitors act through VEGF signaling [37]. Both of these signaling pathways lead to proangiogenic changes through the activation of Ras protein and serine-threonine-specific protein kinases pathways (Raf, MEK, and ERK) [34]. The differences between  $\alpha\beta3$  and  $\alpha\beta5$  occur in the mechanisms of protection against stress and cell death. The  $\alpha\beta3$  stimulation, mediated by bFGF, induces anti-apoptotic processes independent from the ERK mitochondrial activation. In contrast, the  $\alpha\beta5$ /VEGF activation mechanisms are ERG-dependent [38]. Therefore, there is also a possibility that the differences in the secretion of angiogenic cytokines caused by IMPF treatment may be not be related to the expression of  $\alpha\beta3$  integrin. We also observed a decreased expression of CD61 in the reference tissue after IMPF treatment (Table 2). As we previously have described, IMPF administration affects AChE enzyme activity. It is generally known that ACh modulates secretion of several angiogenic factors [39,40]. Altered levels of such angiogenic factors as bFGF and VEGF may in turn impact the expression of integrins both in tumor and healthy tissue.

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## Conclusions

The integrin  $\alpha\beta3$  antagonist inhibited angiogenesis *in vivo* and induced apoptosis in endothelial cells. IMPF has a significant effect on the regulation of lymphocyte-induced angiogenesis, which seems to be related to the modulation of cytokine secretion and pro-inflammatory pro-angiogenic activity.

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