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# THALIDOMIDE AFFECTS THE SKELETAL SYSTEM OF YOUNG RATS

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**Abstract:** Thalidomide has indications for the treatment of several immune-related, neoplastic, and inflammatory diseases, in both adults and children. Despite numerous therapeutic indications for the application of thalidomide, the influence of that drug upon skeletal system has not been recognized. The aim of the present study was to investigate the effects of thalidomide on the osseous tissue in young rats. The experiments were carried out on 5-week-old male Wistar rats. The animals were administered thalidomide in the doses of 15, 30 or 60 mg/kg *p.o.* over the period of 1, 3 or 6 weeks. The body mass gain, bone mass in the tibia, femur and L-4 vertebra, histomorphometric parameters of the femur (width of trabeculae, width of epiphyseal cartilage, the transverse cross-sectional area of the bone marrow cavity and the cortical bone) and the tibia (width of osteoid, diaphysis transverse growth, the transverse cross-sectional area of the bone marrow cavity and the cortical bone) were studied. The investigations carried out provide, for the first time, information concerning the influence of thalidomide upon bone remodeling processes in young rats. The effects of thalidomide on the skeletal system of young rats depended on the dose and upon application time. After administration of doses 15, 30 and 60 mg/kg *p.o.* for 1 and 3 weeks, no influence of thalidomide was noted upon the examined macrometric parameters and histomorphometric parameters of femur, tibia and L-4 vertebra in young rats. Significant disturbances of bone remodeling in young rats have been observed after 6 weeks of thalidomide application, while the progression of those changes increased with the increase of the dose administered. After administering the dose of 15 mg/kg *p.o.* for the period of 6 weeks, no significant changes were found, as regards the macrometric and histomorphometric parameters of bones. Thalidomide, applied 6 weeks in the dose of 30 mg/kg *p.o.,* and in particular in the dose of 60 mg/kg *p.o.*, turned out to disturb bone remodeling processes. In animals administered thalidomide in the dose of 60 mg/kg *p.o.,* reduction mass of tibia, femur, and L-4 vertebra has been observed. In compact bone, thalidomide reduced the diaphysis transverse growth of tibia, reduced the width of osteoid, as well as reduced the transverse cross-sectional area of cortical bone, increased the transverse cross-sectional area of marrow cavity, and increased the transverse cross-sectional area of the marrow cavity/transverse cross-sectional area of the diaphysis ratio of tibia and femur. In cancellous bone, thalidomide reduced the width of bone trabeculae, and increased the width of epiphyseal cartilage. On the basis of the results obtained, one can conclude that thalidomide applied for 6 weeks in the dose of 60 mg/kg *p.o.* inhibited the bone formation processes and increased the bone resorption in young rats.

**Keywords:** bone, rat, thalidomide

Thalidomide was developed in Germany in 1956 as a sedative drug. This agent was shown to have anti-emetic, anxiolytic and adjuvant analgesic properties. Thalidomide has been used to treat morning sickness in pregnant women. In the 1960s and 1970s, scientific reports on teratogenic action of thalidomide were published, which also discussed the related effects of that action, among others defects of osseous system in newborn children. Due to that, after thalidomide had been withdrawn from treatment, studies concerning that drug were discontinued.

At present, thalidomide again generates strong interest, due to the proven anti-inflammatory, immunomodulatory and antitumor action. It has been registered in the USA for treatment of multiple myeloma and erythema nodosum leprosum. Beneficial effect of that drug has also been found in patients with diseases of inflammatory background: ankylosing spondylitis, rheumatoid arthritis, ulcerative colitis, inflammatory bowel disease, Crohn's disease and aphthous stomatitis. The effectiveness of thalidomide has been documented for skin cancer diseases as well as in cachexia and wasting in patients with AIDS, also in numerous dermatological diseases, e.g., lichen planus, sarcoidosis, prurigo nodularis, scleroderma, scleromyxedema, Behcet's disease and numerous others. In recent years, many

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scientific reports came out, on beneficial action of that drug in the graft-*versus*-host disease (GVHD), in neoplasms affecting various organs (kidney, liver, breast, prostate gland, ovary, lung, thyroid gland, pancreas, brain), melanoma, Kaposi's sarcoma, and many other diseases, mainly with inflammatory or autoimmune background (1-3).

The mechanism of thalidomide action is varied and multidirectional, on the molecular level it has not been completely recognized. The anti-inflammatory action of thalidomide is related to the inhibition of synthesis and release of pro-inflammatory cytokines, mainly tumor necrosis factor-α (TNF-α) (4, 5), interleukin-1 (IL-1) (6) and interleukin-6 (IL-6) (7).

The results of *in vitro* and *in vivo* studies indicate that the main mechanism of immunomodulatory action of thalidomide results from inhibition of production and release of TNF-α in monocytes (8). That drug also activates T lymphocytes and increases their proliferation (9). Thalidomide increases the number of NK cells and increases their cytotoxic properties (10). Under the influence of thalidomide, the number of B lymphocytes increases (11). Other studies indicate that thalidomide induces mechanisms which block receptors for integrins and other adhesins (12). Moreover, that drug reduces the generation of IgM (13).

Thalidomide occurs to be most effective in neoplastic diseases, which have substantial angiogenesis, e.g., multiple myeloma. The drug arrests angiogenesis as a result of inhibiting the angiogenic factors - basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) (14), as well as reduced synthesis and release of TNF-α and IL-6 (15). Thalidomide exerts direct anti-proliferative effect, as a result of inhibiting the growth of neoplastic cells in the $G_1$ phase of cell cycle (16). The drug also enhances the apoptosis of neoplastic cells. It has been demonstrated that attenuation, by thalidomide, of the transcription activity of nuclear factor κB (NF-κB) leads to inhibition of gene expression for cellular inhibitor of apoptosis-2 (cIAP-2) (17). In the mechanism of antineoplastic action of thalidomide, also the influence of the drug upon adhesins is taken into account – namely intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). As a result, the mutual adherence of neoplastic cells is weakened (1, 15). In patients with multiple myeloma the therapeutic effect of thalidomide is a result of inhibiting the activity of IL-6 instroma cells of bone marrow (1, 15). The immunomodulating influence of thalidomide in neoplastic diseases is also connected with an increase of the number and enhancing the cytotoxic properties of NK cells (18), that contributes to intensified anti-neoplastic response of the immunological system.

For twenty years now, research has been carried out to obtain immunomodulatory drugs (IMDs) of the second generation, as well as a new category of drugs, the so-called selective cytokine inhibitory drugs (SelCIDs). Those compounds are derivatives of thalidomide. IMDs, besides thalidomide, comprise also lenalidomide and pomalidomide. They have a similar mechanism of anti-inflammatory and antitumor action, as the mother drug (19).

For a few years, there have been attempts to use thalidomide in pediatric practice. Therapeutic properties of that drug have been noted in children suffering from juvenile idiopathic arthritis, Behcet's disease, inflammatory calcinosis, bowel disease, Crohn's disease, sarcoidosis, aphthous stomatitis, dyskeratosis, GVHD, tuberculosis of the central nervous system, leukemia, tumors of thyroid, tumors of central nervous system, and others (20-35). As concerns the analogs of thalidomide, also the application of lenalidomide in oncological treatment of children is considered (36).

Despite the beneficial effect in the treatment of numerous diseases, thalidomide also has side-effects. Among the commonly reported there are: peripheral neuropathy, somnolence and constipation. In patients treated with thalidomide, the following symptoms are also observed: leucopenia, headache, depression, intermittent shaking, increased appetite, nausea, brittle finger nails, dry mouth, pruritus, pustuloderma (2, 20, 27, 37).

The security of applying thalidomide as regards skeletal system has not been determined as yet. In order to provide the explanation of that problem, the study reported here has investigated the influence of thalidomide upon bone remodeling processes in young rats.

## EXPERIMENTAL

The experiments were carried out on young (5-week-old) maleWistar rats, which were fed a standard diet *ad libitum*. The experimental procedures were approved by the Local Ethics Commission, Katowice, Poland. Thalidomide [(±)-2-(2,6-dioxo-3-piperidinyl)-1H-isoindole-1,3(2H)-dione] was purchased from Sigma-Aldrich.

The animals were divided into 10 groups (n = 7): I – control rats; II – rats receiving thalidomide 15 mg/kg *p.o.* for 1 week; III – rats receiving thalidomide 30 mg/kg *p.o.* for 1 week; IV – rats receiving

thalidomide 60 mg/kg *p.o.* for 1 week; V – rats receiving thalidomide 15 mg/kg *p.o.* for 3 weeks; VI – rats receiving thalidomide 30 mg/kg *p.o.* for 3 weeks; VII – rats receiving thalidomide 60 mg/kg *p.o.* for 3 weeks; VIII – rats receiving thalidomide 15 mg/kg *p.o.* for 6 weeks; IX – rats receiving thalidomide 30 mg/kg *p.o.* for 6 weeks; X – rats receiving thalidomide 60 mg/kg *p.o.* for 6 weeks.

The animals were given tetracycline hydrochloride(20 mg/kg, *i.p.*) to mark the calcification front one day before administration of the drug began, and on the last day of thalidomide administration. The day following the last administration of the drug, the animals were anesthetized and sacrificed. The tibias, femurs and L-4 vertebra were excised and weighed. In the long bones isolated from the left side, the macrometric parameters were determined (length, diameter of the diaphysis at mid-length and diameter of the epiphysis).

The femoral and tibial bones were used to prepare histological specimens. The histological specimens were prepared and measured as previously described (38, 39). In the longitudinal preparation from the femur, the width of epiphyseal cartilage and the width of trabeculae in the epiphysis and metaphysis were measured. The area of the transverse cross-section of the cortical bone and the area of the transverse cross-section of the marrow cavity were determined in transverse cross-sections made from the diaphysis at the mid-length of the femur and the tibia. The periosteal and endosteal transverse growth and the width of the periosteal and endosteal osteoid were measured in transverse cross-sections made from the tibial diaphysis.

Histomorphometric measurements were made using an Optiphot-2 microscope (Nikon), connected through an RGB camera (Cohu) to a personal computer (software: Lucia G 4.51, Laboratory Imaging), with final magnifications of 200× and 500×, and a lanameter (magnification 50×).

Additionally, body mass gain, mass of the liver, spleen and thymus were studied.

The results are presented in tables as the arithmetic mean ± SEM. The results obtained in the rats receiving thalidomide were compared to the results of the control rats with the use of one-way ANOVA followed by Dunnett's *post-hoc* test.

Table 1. Effects of thalidomide administered for 6 weeks on bone macrometric parameters in young rats.

| Bone macrometric parameters | **Control** | **THALIDOMIDE** | | | ANOVA |
|---|---|---|---|---|---|
| | | **15 mg/kg *p.o.*** | **30 mg/kg *p.o.*** | **60 mg/kg *p.o.*** | |
| **FEMUR** | | | | | |
| Mass [mg] | 700.55 ± 17.15 | 641.93 ± 18.14 | **641.93 ± 18.14** * | **619.90 ±15.99** ** | $p < 0.05$ |
| Length [mm] | 34.12 ± 0.61 | 33.98 ± 0.40 | 33.82 ± 1.31 | **30.87 ± 1.57** | NS |
| Diameter of the diaphysis [mm] | 3.80 ± 0.09 | 4.00 ± 0.09 | 4.06 ± 0.09 | 3.91 ± 0.06 | NS |
| Diameter of the epiphysis [mm] | 6.58 ± 0.08 | 6.43 ± 0.11 | 6.47 ± 0.10 | 6.44 ± 0.28 | NS |
| **TIBIA** | | | | | |
| Mass [mg] | 513.55 ± 7.51 | 523.07 ± 19.72 | **441.82 ± 30.40** * | **433.82 ± 32.49** * | $p < 0.05$ |
| Length [mm] | 37.23 ± 0.48 | 36.18 ± 1.19 | 35.94 ± 1.41 | **35.83 ± 2.17** | NS |
| Diameter of the diaphysis [mm] | 3.13 ± 0.08 | 3.12 ± 0.05 | 3.23 ± 0.11 | 3.23 ± 0.13 | NS |
| Diameter of the epiphysis [mm] | 6.20 ± 0.13 | 6.22 ± 0.13 | 6.21 ± 0.16 | 6.18 ± 0.14 | NS |
| **L-4 VERTEBRA** | | | | | |
| Mass [mg] | 203.42 ± 8.03 | 210.10 ± 2.92 | **166.0 ±8.39*** | **161.03 ± 19.00** * | $p < 0.01$ |

Results are presented as the means ± SEM (n = 7). The experiments were carried out on 5-week-old male rats (at the start of thalidomide administration). The results obtained in the thalidomide group were compared with those of the control rats using one-way ANOVA followed by Dunnett's *post-hoc* test. NS – no statistically significant differences. * – $p < 0.05$; ** – $p < 0.01$ – statistically significant differences in comparison with the control rats.

## RESULTS

The results are presented in Tables 1 and 2.

Thalidomide given at doses of 15, 30 or 60 mg/kg *p.o.* for the period of 1 week did not affect the macrometric and histomorphometric parameters. Likewise, the application of thalidomide for 3 weeks did not cause significant changes of the investigated parameters.

Administration of thalidomide at doses of 30 mg and 60 mg/kg *p.o.* for 6 weeks affects the skeletal system of young rats.

In rats receiving thalidomide at a dose of 30 mg/kg *p.o.*, there were statistically significant decreases in the bone mass of the femur, tibia and L-4 vertebra (by 8.4%, 14.0% and 18.2%, respectively) compared to control rats.

Thalidomide given at a dose of 30 mg/kg *p.o.* for the period of 6 week did not affect the length, the diameter of the diaphysis and the diameter of the epiphysis, in both femur and tibia, in comparison with the rats of the control group.

In rats which were administered thalidomide at a dose of 30 mg/kg *p.o.*, the following changes in the femur were found in comparison with the rats of the control group: a decrease in transverse cross-sectional area of the cortical bone by 7.7%, an increase in the transverse cross-sectional area of the marrow

Table 2. Effects of thalidomide administered for 6 weeks on bone histomorphometric parameters in young rats.

| Bone histomorphometric parameters | | Control | THALIDOMIDE | | | ANOVA |
|---|---|---|---|---|---|---|
| | | | **15 mg/kg *p.o.*** | **30 mg/kg *p.o.*** | **60 mg/kg *p.o.*** | |
| **FEMUR** | | | | | | |
| Width of trabeculae [μm] | Epiphysis | 56.02 ± 2.55 | 57.72 ± 0.34 | 55.87 ± 2.03 | **52.70 ± 2.65** | NS |
| | Metaphysis | 40.95 ± 1.06 | 42.67 ± 2.63 | 42.62 ± 3.05 | **38.55 ± 1.95** | NS |
| Width of epiphyseal cartilage [μm] | | 69.04 ± 2.51 | 65.14 ± 1.69 | 66.32 ± 1.94 | **79.14 ± 2.94** * | $p < 0.05$ |
| Transverse cross-sectional area [$mm^2$] | Cortical bone | 3.64 ± 0.12 | 3.48 ± 0.12 | **3.36 ± 0.05** | **3.26 ± 0.21** | NS |
| | Marrow cavity | 1.08 ± 0.05 | 1.09 ± 0.10 | **1.18 ± 0.06** | **1.27 ± 0.04*** | $p < 0.05$ |
| | Diaphysis | 4.73 ± 0.08 | 4.57 ± 0.18 | **4.54 ± 0.06** | 4.53 ± 0.22 | NS |
| | Ratio# | 0.23 ± 0.01 | 0.24 ± 0.02 | **0.26 ± 0.01** | **0.28 ± 0.0*** | $p < 0.05$ |
| **TIBIA** | | | | | | |
| Diaphysis transverse growth [μm] | Periosteal | 144.32 ± 4.67 | 138.99 ± 7.47 | **127.91 ± 7.23** | **111.28 ± 10.34** * | $p < 0.05$ |
| | Endosteal | 66.07 ± 1.67 | 61.93 ± 4.76 | **55.51 ± 6.19** | **48.50 ± 4.39** ** | $p < 0.05$ |
| Width of osteoid [μm] | Periosteal | 16.46 ± 0.78 | 16.74 ± 0.47 | **14.06 ± 0.94** | **11.73 ± 1.04** ** | $p < 0.01$ |
| | Endosteal | 10.34 ± 0.62 | 11.27 ± 0.58 | **9.05 ± 0.77** | **7.55 ± 1.08** * | $p < 0.05$ |
| Transverse cross-sectional area [$mm^2$] | Cortical bone | 3.121 ± 0.045 | 3.247 ± 0.055 | **2.694 ± 0.214** | **2.863 ± 0.159** | NS |
| | Marrow cavity | 1.008 ± 0.087 | 1.034 ± 0.022 | **1.188 ± 0.052** | **1.408 ± 0.086** ** | $p < 0.01$ |
| | Diaphysis | 4.130 ± 0.067 | 4.281 ± 0.049 | 3.882 ± 0.227 | 4.201 ± 0.110 | NS |
| | Ratio# | 0.243 ± 0.017 | 0.242 ± 0.006 | **0.310 ± 0.019** * | **0.332 ± 0.025** * | $p < 0.01$ |

**#** – the transverse cross-sectional area of the marrow cavity/transverse cross-sectional area of the diaphysis ratio. Results are presented as the means ± SEM (n = 7). The experiments were carried out on 5-week-old male rats (at the start of thalidomide administration). The results obtained in the thalidomide group were compared with those of the control rats using one-way ANOVA followed by Dunnett's *post-hoc* test. NS – no statistically significant differences. * - $p < 0.05$; ** - $p < 0.01$ – statistically significant differences in comparison with the control rats.

cavity by 9.3% and an increase in the transverse cross-sectional area of the marrow cavity/transverse cross-sectional area of the diaphysis ratio by 13.0%. In the tibia, in comparison with the control group, a decrease in the transverse cross-section area of the cortical bone by 13.7%, an increase in the transverse cross-section area of the marrow cavity by 17.8% and a significant increase in the transverse cross-sectional area of the marrow cavity/transverse cross-sectional area of the diaphysis ratio by 27.6%, were observed.

In comparison with the control group, the following changes were observed in tibial bone of rats which were administered thalidomide at a dose 30 mg/kg *p.o.*: reduced diaphysis transverse growth on the periosteum side (by 11.4%) and on the endosteum side (by 16.0%), as well as reduced width of the periosteal osteoid (by 14.6%) and endosteal osteoid (by 12.5%).

Thalidomide given at a dose of 30 mg/kg *p.o.* for the period of 6 week did not affect the width of trabeculae or the width of epiphyseal cartilage in comparison with the rats of the control group.

Administration of thalidomide at a dose of 60 mg/kg *p.o.* for the period of 6 weeks caused a significant decrease in the mass of femur by 11.5%, the mass of tibia by 15.5% and the mass of L-4 vertebra by 20.9%. The decrease in the length of the femoral bone by 9.4% and tibial bone by 3.5% was observed. Thalidomide given at a dose of 60 mg/kg *p.o.* for the period of 6 weeks did not affect the femoral and tibial diameter of the diaphysis and the diameter of the epiphysis, in comparison with the rats of the control group.

The width of trabeculae in the epiphysis and metaphysis in rats receiving thalidomide at a dose of 60 mg/kg *p.o.* decreased by 5.9% in comparison with the controls. Thalidomide in the femoral bone decreased the transverse cross-section area of the cortical bone (by 10.4%), increased in the transverse cross-section area of the marrow cavity (significantly, by 17.6%) and increased the transverse cross-sectional area of the marrow cavity/transverse cross-sectional area of the diaphysis ratio (significantly, by 21.7%). The width of epiphyseal cartilage of the femur was narrower than in the control group, by 14.6% (significantly).

In rats which were administered thalidomide at a dose of 60 mg/kg *p.o.*, the following changes in the tibia were found in comparison with the rats of the control group: a decrease in transverse cross-sectional area of the cortical bone by 8.3%, a significant increase in the transverse cross-sectional area of the marrow cavity by 39.7% and a significant increase in the transverse cross-sectional area of the marrow cavity/transverse cross-sectional area of the diaphysis ratio by 36.4%. A significant decrease in the diaphysis transverse growth on the *periosteum* side and on the *endosteum* side (by 22.9% and 26.6%, respectively) and significantly reduced width of the periosteal osteoid (by 28.7%) and endosteal osteoid (by 27.0%) was observed, compared to control rats. Thalidomide given at a doses of 15, 30 and 60/kg *p.o.* for the period of 1, 3 or 6 weeks did not affect the body mass and mass of liver, thymus and spleen (data not shown).

## DISCUSSION

At present, thalidomide, because of its well proven and strong anti-inflammatory, antitumor, and immunomodulating action, ranks among the most thoroughly investigated drugs worldwide. However, the influence of thalidomide upon the osseous system has not been the subject of specific clinical studies, neither in adults nor in children. The application of thalidomide in treatment of many diseases calls for carrying out studies pertaining to the side-effects within the osseous system.

Literature data refer mainly to the teratogenic influence of thalidomide upon the osseous tissue in fetuses of laboratory animals, and in children born from mothers who took thalidomide during pregnancy. The mechanism of teratogenic effect of thalidomide upon the osseous system still remains unexplained. Taken into consideration are the following: influence of the drug upon DNA replication and transcription, upon angiogenesis and chondrogenesis, as well as upon synthesis and functioning of integrins and growth factors. The teratogenic action of thalidomide results mainly from inhibition of angiogenesis, which is particularly intensified in developing embryo. That is why in ”thalidomide babies” the ischemia of limb buds occurred, followed in consequence by ectromelia. An important role in that process is played by TNF-α. That cytokine activates the angiogenic factors, namely bFGF and VEGF. Thalidomide, by blocking TNF-α, in consequence inhibits angiogenesis. Irrespective of that action, the drug may also directly inhibit the activity of angiogenic factors bFGF and VEGF (40, 41).

However, the research in recent years has indicated beneficial influence of thalidomide in osteolytic changes in bones of patients suffering from multiple myeloma (42-44). Thalidomide, administered to experimental animals, prevented intensification of resorption processes in bones with ovariecto-

my- or glucocorticoid-induced osteoporosis and osteomyelitis aseptica (45-47).

Scarce clinical and experimental data concerning the influence of thalidomide upon osseous tissue, as well as the possibilities of multidirectional application of that drug substantiate the need of recognizing the influence of thalidomide on bone remodeling processes, both in adults and in children. In order to examine the effect of thalidomide on the osseous tissue in young organisms, thalidomide was administered to young (5-week-old) male rats. The drug was administered to rats once a day *per os*, for the period of 1, 3 or 6 weeks. The effective doses of thalidomide, used in clinical studies in children vary between 1.5 mg/kg/day and 6 mg/kg/day (maximum up to 12 mg/kg/day) (20-35), however, the therapeutic dose of the drug to be used in pediatric practice has not been firmly determined. That is why thalidomide has been applied to young rats in three different doses: 15, 30 and 60 mg/kg. Systemic metabolism in rats is intensified, in comparison with that in humans, thus the tenfold conversion factor has been applied for the dosage of thalidomide. The same doses had been used in earlier studies of the influence of thalidomide upon osseous tissue in adult rats (47), as well as in investigation of that drug's influence upon ovariectomy- or glucocorticoid-induced osteoporosis in rats (45, 46). Locker et al. stated in their report that acute toxicity of thalidomide ($LD_{50}$) determined in young 3-week-old rats after oral administration, amounts to more than 8 g/kg of body mass (48).

Femur and tibia belong to long bones. The diaphysis of long bones is made of compact bone tissue. The epiphysis and metaphysis of these bones are built mainly of cancellous bone tissue. Compact bone tissue and cancellous bone tissue differ by location in the skeletal system, their internal microarchitecture and the physiological function they perform. In this study, such differences between the types of osseous tissue were the basis to assess the bone remodeling processes based on histomorphometric parameters of compact bone tissue (marking increments in diaphysis transverse growth of tibia, the transverse growth cross-sectional area of cortical part of femur and tibia, the transverse growth in cross-sectional area of marrow cavity of femur and tibia and the transverse growth ratio in cross-sectional area of the marrow cavity/transverse cross-sectional area of the diaphysis of these bones), as well as in cancellous bone tissue (marking the width of trabeculae of femur metaphysis and epiphysis, and the width of epiphyseal cartilage in femur). The examined histomorphometric parameters of bones, including macrometric parameters, allow for assessing bone remodeling, which consists of both bone formation and bone resorption.

The effects of thalidomide on the skeletal system of young rats depended on the dose and period of administration. After administration of doses of 15, 30 and 60 mg/kg *p.o.* over the period of 1 and 3 weeks, no influence of thalidomide was noted upon the examined macrometric parameters and histomorphometric parameters of femur, tibia and L-4 vertebra in young rats.

Substantial disturbances of bone remodeling in young rats have been noted after 6 weeks of thalidomide administration, while the progression of those changes intensified with the increase of dose applied. After administration of 15 mg/kg *p.o.* doses for the period of 6 weeks, no significant changes have been found to occur in the examined macrometric and histomorphometric parameters of bones, in comparison with control. In the experiment, in which young rats were administered the dose of 30 mg/kg *p.o.* statistically significant reduction of femur mass was found, as well as statistically significant reduction of tibia mass, and the L-4 vertebra mass. In those animals, the length and diameter of femur and tibia shafts remained unchanged. The result of inhibiting the bone formation was a reduction of periosteal and endosteal diaphysis transverse growth, as well as reduction of synthesis of the non-calcified bone matrix (decreased width of periosteal and endosteal osteoid) in tibia. Osteoid is an organic part of bone matrix, synthesized mainly by osteoblasts in the bone formation process. Osteoid undergoes mineralization in further stages of bone formation. A decrease in the width of osteoid may indicate a decrease in forming of the organic part of bones and, thus, inhibition of bone formation processes.

Reduction of the transverse cross-sectional area of cortical bone, an increase of the transverse cross-sectional area marrow cavity, as well as an increase of the transverse cross-sectional area of the marrow cavity/transverse cross-sectional area of the diaphysis ratio, both in femur and tibia, may indicate intensified bone resorption in young rats receiving thalidomide in the dose of 30 mg/kg *p.o.* However, since influence of this drug on the width of trabeculae in epiphysis and metaphysis of femur was not previously observed, this effect is unlikely to be occured. Cancellous bone, having a substantial surface, well supplied with blood, is particularly exposed to the activity of resorption factors. Moreover, young individuals are characterized by high level of bone turnover, while bone formation prevails over bone resorption (49).

After 6 weeks of administration of thalidomide in young rats, at the highest dose of 60 mg/kg *p.o.,* more significant disturbances in bone remodeling process have been noted, in comparison with rats administered the same drug in the dose of 30 mg/kg *p.o*. In those animals, statistically significant reduction of mass of the femur, tibia, and L-4 vertebra has been noted, in comparison with control. In compact bone, thalidomide – after 6 weeks of administration in the dose of 60 mg/kg *p.o.,* inhibited the formation process. That has been certified by statistically significant reduction of periosteal and endosteal diaphysis transverse growth of tibia, including statistically significant decrease of the width of periosteal and endosteal osteoid, as well as statistically significant reduction of the transverse cross-sectional area of cortical bone of tibia and femur. Also, intensified resorption processes have been found in compact bones, which has been proven by statistically significant increase of transverse cross-sectional area of marrow cavity, as well as statistically significant increase of the transverse cross-sectional area of the marrow cavity/transverse cross-sectional area of the diaphysis ratio in femur and in tibia.

In cancellous bone in turn, that is in femoral epiphysis and metaphysis, thalidomide administered to rats at the dose of 60 mg/kg *p.o.* for 6 weeks caused reduction of the width of bone trabeculae. Those changes may result both from inhibition of bone formation and from intensification of the resorption process. In femur, also statistically significant increase of the width of epiphyseal cartilage has been observed, and – related to it – reduced length of that bone, in comparison with control animals. A similar result has been obtained in case of measurements of tibial length. Epiphyseal cartilage in the long bone is a place where cell multiplication takes place, as well as bone lengthening. During osseus system development in human, the epyphisis cartilage lessens, and when individual matures, then it wanes. In rats, the epiphyseal cartilage never vanishes, as a rat keeps growing throughout its lifetime, that is why rats are a good experimental specie for the assessment of the influence of drugs upon lengthening of bones.

Because thalidomide demonstrates immunomodulatory properties, the assessment also included spleen mass and thymus mass, because those organs are part of the immunological system. Irrespective of the dose and period of administration of thalidomide, no substantial changes have been revealed, pertaining to spleen or thymus mass in young rats. Likewise, no change occurred as regards the mass of liver or body mass, in comparison with control. The above is confirmed by literature data, where Locker et al., having administered thalidomide in doses of 10 and 50 mg/kg, intraperitoneally to 3-week-old rats, failed to find any changes in body mass or mass of internal organs, apart from an increase of the mass of adrenal glands (48).

Summing up, it can be stated on the basis of macrometric and histomorphometric measurements that administration of thalidomide at a dose of 30 mg/kg *p.o*., and in particular at a dose of 60 mg/kg *p.o.,* for the period of 6 weeks, results in disturbed bone remodeling in young rats. Changes in the osseous system of young rats are probably a result of reduced bone formation and increased bone resorption process.

Similar results had been obtained in earlier studies performed on mature rats, in which it had been found that thalidomide used in the dose of 60 mg/kg *p.o.* for 6 weeks had destructive influence upon osseous tissue. The drug occurred to disturb the reconstruction processes by inhibiting the formation process, including mineralization, and intensified bone resorption (47).

In that phase of studies on the effect of thalidomide on osseous system of young rats, it is difficult to define the mechanism of negative impact of that drug on bone remodeling. In culture of osteoblasts in mice, no influence of thalidomide upon mRNA expression of factors responsible for bone formation - $\alpha$2 procollagen I, osteoprotegerin, as well as factors that regulate bone resorption – ligand receptor for activation of nuclear factor $\kappa$B (RANKL), macrophage colony-stimulating factor (M-CSF), TNF-$\alpha$, IL-1, IL-6, as well as prostaglandin $E_2$ synthase. Those results indicate that the above factors do not participate in disturbing bone remodeling by thalidomide in rats (47).

On the other hand, stimulation of resorption processes by thalidomide has been confirmed by the results of *in vitro* studies, which revealed the formation of osteoclasts in cultures of mouse bone marrow cells. Thalidomide intensified osteoclastogenesis in concentration-dependent manner. In the concentration of $10^{-3}$ M the drug caused an increase of the number and diameter of mature osteoclasts, as well as increase of the total number of TRAP-positive osteoclasts. In the concentrations of $10^{-4}$ M and $10^{-5}$ M thalidomide only increased the amount of TRAP-positive osteoclasts, and had no influence upon the amount of mature osteoclasts. In the concentration of $10^{-6}$ M, no influence of thalidomide was observed upon proliferation of osteoclasts (47). However, the *in vitro* studies performed by Anderson et al. (50) demonstrated inhibition of osteoclastogenesis by

thalidomide. In the concentrations of 1, 25, 50, 75 and 100 μM thalidomide inhibited the formation of osteoclasts by 28.5, 50, 52, 57.1 and 64.3%, respectively. According to the researchers, the mechanism of osteoclastogenesis reduction by thalidomide has not been connected with inhibition of transcription factors: c-fos and PU.1 (inducing the formation of osteoclast precursors). Colla et al. demonstrated that thalidomide did not attenuate the expression of DKK-1, a protein inhibiting the factor responsible for the expression of genes responsible for differentiation of osteoblasts (51).

It should be mentioned that – as demonstrated in the studies on rats with disturbed bone remodeling processes, thalidomide had positive action. Thalidomide prevents disturbances of bone remodeling in ovariectomized rats (45), rats with osteomyelitis aseptica (47) and in glucocorticoid-induced osteoporosis in rats (46). In the recent years, clinical trials also have indicated the positive impact of thalidomide and its analogs in osteolytic changes of bones in patients with multiple myeloma (42-44).

## CONCLUSIONS

The research conducted provided, for the first time, the information concerning the influence of thalidomide upon bone remodeling processes in young rats. It has been demonstrated that thalidomide administered for 6 weeks at the dose of 30 mg/kg *p.o.*, and in particular at the dose of 60 mg/kg *p.o.*, disturbed the bone remodeling processes. The drug turned out to inhibit the bone formation processes and to intensify the resorption of osseous tissue in young rats.

The presented results of the study provide the basis for performing clinical observations as to the side-effects of thalidomide within the osseous system in humans, in particular in children.

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