Hsp90 and Angiogenesis in Bone Disorders – Lessons from the Avian Growth Plate

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Thiram-induced tibial dyschondroplasia (TD) and vitamin-D deficiency rickets are avian bone disorders of different etiologies characterized by abnormal chondrocyte differentiation, enlarged and unvascularized growth plates, and lameness. Heat-shock protein 90 (Hsp90) is a pro-angiogenic factor in mammalian tissues and in tumors, therefore, Hsp90 inhibitors were developed as anti-angiogenic factors. In this study we evaluated the association between Hsp90, hypoxia, and angiogenesis in the chick growth plate.

Administration of the Hsp90 inhibitor to TD- and rickets-afflicted chicks at the time of induction resulted in reduction in growth-plate size and, contrary to its anti-angiogenic effect in tumors, a major invasion of blood vessels occurred in the growth plates. This was the result of up-regulation of the vascular endothelial growth factor (VEGF) receptor Flk-1, the major rate-limiting factor of vascularization in TD and rickets. In addition, the abnormal chondrocyte differentiation, as characterized by collagen type II expression and alkaline phosphatase activity, and the changes in hypoxia-inducible factor-1α (HIF-1α) in both disorders were restored. All these changes resulted in prevention of lameness. Inhibition of Hsp90 activity reduced growth-plate size, increased vascularization, and mitigated lameness also in TD chicks with established lesions.

In summary, this is the first reported demonstration of involvement of Hsp90 in chondrocyte differentiation and growth-plate vascularization. In contrast to the anti-angiogenic effect of Hsp90 inhibitors observed in mammals, inhibition of Hsp90 activity in the unvascularized TD- and rickets-afflicted chicks resulted in activation of the angiogenic switch and reinstated normal growth-plate morphology.
LONGITUDINAL BONE GROWTH is formed by endochondral ossification that occurs in the growth plates located at the ends of long bones. The resting chondrocytes of the growth plate proliferate slowly and then accelerate their rate of proliferation, divide rapidly along the longitudinal axis of the bone, and form characteristic columns of cells. Ultimately, the most distal cells of the columnar layer stop proliferating, exit the cell cycle, and differentiate into hypertrophic chondrocytes: a change that is characterized by changes in the gene expression profile and by an increase in cell volume. Proliferation and hypertrophy of chondrocytes, along with ECM synthesis, are the main forces of endochondral bone growth (2,19,22,27). The hypertrophic chondrocytes mineralize their ECM and undergo apoptosis or autophagy, and the area of hypertrophic cartilage, along with bone precursor cells, is invaded by blood vessels (6,7,34). The capillary invasion mediated by vascular endothelial growth factor (VEGF) is a key mechanism for the precise coupling of chondrogenesis and osteogenesis that determines the rate of bone growth and is a prerequisite for bone formation (11,12). Any changes in this balance might induce pathological conditions, as exemplified by the large numbers of human chondrodysplasias and of transgenic mice (15,19). Production of VEGF by the hypertrophic chondrocytes is regulated by hypoxia and by the hypoxia-inducible factor-1α (HIF-1α) (1,31,32,43,44). HIF-1α is the major regulator of the hypoxic responses such as chondrocyte growth arrest, survival, maturation, and apoptosis that are essential for chondrogenesis and that mediate oxygen-dependent changes in glucose uptake, increases in red blood cell production, and formation of new blood vessels via angiogenesis (4,14,27,31,37). HIF-1α is one of the major client proteins of heat-shock protein 90 (Hsp90), and it is required for the functioning and the rapid hypoxic stabilization of HIF-1α, which otherwise is degraded by the ubiquitin-proteasome protein system (17). Hsp90 is implicated in angiogenesis by affecting the VEGF/VEGF-receptor system at various levels (5,35,45) and various inhibitors of Hsp90 activity are being evaluated in clinical trials as anti-angiogenic factors (21,29). Modulation of HIF-1 activity is highly dependent on HSP90 inhibitor concentration. An increase in HIF-1α protein levels as well as HIF target gene expression such as VEGF was found in the low nanomolar range, whereas higher doses efficiently down-regulated the HIF system (16).
Compared with the mammalian growth plate, the avian one contains much longer columns of chondrocytes, more cells are found in each zone, and the metaphyseal blood vessels penetrate deeply into the growth plate, thereby making the avian growth plate much more vascular than the mammalian one (24,25). Although an oxygen-related gradient was observed within the chick growth plate, no hypoxia was detected, and the oxygen status of the cells throughout the cartilage was consistent with their oxygen needs (33). Thiram-induced tibial dyschondroplasia (TD) and vitamin-D deficient rickets (Vit D-) are avian bone disorders characterized by enlarged and unvascularized growth plates, and lameness (3,26). The first is the result of copper deficiency; the other is the result of reductions in plasma calcium and phosphorus because of vitamin-D deficiency. Dyschondroplasia was attributed to abnormal differentiation of chondrocytes (26), to changes in expression of genes encoding VEGF signaling (30), and to changes in matrix metalloproteinase (MMPs) activities (9,13).

In the present study we evaluated the association between Hsp90, hypoxia, and angiogenesis in the chick growth plate. Two growth-plate disorders of dissimilar etiologies and with differing hypoxia status, but both causing a major reduction in vascularization were used. By inhibition of Hsp90 activity and \textit{in situ} evaluation of chondrocytes differentiation, hypoxia, HIF-1\(\alpha\) gene expression, and of the VEGF and its receptor Flk-1 we were able to establish, for the first time, the role of Hsp90 in growth-plate differentiation and vascularization.

**MATERIALS AND METHODS**

\textit{Growth plate disorders and hypoxia determination}

Day-old male broiler chicks (Cobb strain) were raised in battery brooders at 24°C. Dyschondroplasia was induced by dietary thiram at 40 ppm in feed from day 3, and rickets by a vitamin D-deficient diet starting at day 1 post-hatch (3). Development of TD lesions (TDL) was verified on day 10. Growth-plate hypoxia was evaluated \textit{in situ} by injection of Hypoxyprobe (Hypoxyprobe-1, Chemicon International) into the wing vein at 40mg/kg as previously described (10). In the prevention protocol TD was induced by 40 ppm thiram-containing diet at day 4 post-hatch until day 10 at the time the chicks were sacrificed. The geldanamycin analog 17-DMAG (17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (InvivoGen), a specific inhibitor of Hsp90 activity (39), was administered into the wing vein (600\(\mu\)g) at days
3 (one day before TD induction) and at day 5. Rickets was induced by a vitamin D-
deficient diet from the first day post-hatch and 17-DMAG (600μg) was administered
at days 3 and 7 and chicks were sacrificed at day 12. In the treatment protocol, TD
was induced by a diet containing 25ppm thiram, 17-DMAG (600μg) was administered
at days 7, 10 and 12 post-hatch and chicks were sacrificed at day 14. All control
chicks were administered with saline.

This dose was selected based on studies using 17-DMAG to prevent tumor
development in mice. All animal experiments were carried out according to the
guidelines of the Volcani Center Institutional Committee for Care and Use of
Laboratory Animals.

*Growth plate sections, immunohistochemistry and in situ hybridization*

Immediately after sacrifice by cervical dislocation, the tibiae were fixed in 4%
paraformaldehyde (3). Immunohistochemistry was performed with VEGF147 and Flk-1
rabbit polyclonal antibodies (1:50, Santa Cruz Biotechnology), HIF-1α mouse
monoclonal (1:250, Novus Biologicals), and α smooth-muscle actin (αSMA)
monoclonal antibodies (1:200, Dako). Alkaline phosphatase (AP) staining was
performed as previously described (18). For *in situ* hybridization the avian
PTH/PTHrP receptor and collagen type II probes were used (3,42). The levels of
VEGF and Flk-1 were analyzed with ImagePro software (Media Cybernetics, Inc.,
Silver Spring, MD). Photographs of 8 sections from each growth plate from 3
different chicks from each group were taken for analysis. The results are presented as
arbitrary units of the mean ± SE.

*Bone ash, plasma calcium, phosphorus and 25(OH)D₃ determination*

Calcium and phosphate were analyzed by COBAS INTEGRA® 400 plus and ionic
calcium by Omni (Roche Diagnostics), 25-hydroxyvitamin D₃ (25(OH)D₃) was
analyzed by 2-site chemiluminescence assay (38), and bone ash was determined as
described by Yalcin et al.(46).

*Cultured chondrocytes*

Primary avian epiphyseal growth-plate chondrocytes prepared as previous described
(3) were incubated for 3 days with 0.5 μM 17-(allylamino)-17-
demethoxygeldanamycin (17-AAG, EMD Chemicals). 17-AAG is highly potent *in*
vitro but with poor pharmaceutical properties such as aqueous solubility, stability, hepatotoxicity and formulation difficulties while 17-DMAG which retained potent Hsp90 inhibition has improve aqueous solubility and improved pharmacologic profile. The primers for real-time PCR were: for HIF-1α, 5’-CCGTCAAATCGAAACAACTTT-3’ 5’-TGTATGGGACTCACTCAGGTGAA-3’ and for Glut-1, 5’-GAGCCAATGGTGGCGTAGAC-3’ 5’-GGATCAATGCGGTTTTCTACTACTC-3’.

Statistical analysis

One-way ANOVA was applied and means were separated by Tukey’s test when appropriate. The level of significance used in all results was P < 0.05.

RESULTS

Inhibition of Hsp90, chondrocyte differentiation, and growth-plate histopathology

Dyschondroplasic and vit D- chicks exhibited enlarged and unvascularized growth plates that resulted in abnormal bone growth, reduced BW and lameness (Fig. 1 and Table 1). Administration of 17-DMAG to the TD-afflicted chicks prevented the increase in growth-plate width, which at day 10 did not differ from that of the controls. In addition, BW was partially restored and the chicks regained their ability to stand and walk properly. In the vit D- chicks, although no changes in BW were observed after 17-DMAG administration, the growth plates were significantly smaller and the chicks did not exhibit any signs of lameness. These improvements were probably the result of a local effect of 17-DMAG on growth-plate Hsp90 activity: no changes in plasma calcium (total or ionic), phosphorus or 25-hydroxyvitamin D₃ were observed and bone ash was unaltered (Table 1). Both disorders are characterized by abnormal differentiation of chondrocytes along the bone axis (Fig. 2). Collagen type II is synthesized by chondrocytes of the proliferative zone; it is up-regulated in TD, especially above the lesion, and is unaffected in rickets. In the present study, in both cases, collagen type II gene expression was reduced after 17-DMAG treatment and in TD reached control levels. The AP activity that characterizes hypertrophic chondrocytes is absent within the TD lesion, and in rickets chondrocytes that exhibit AP activity are located at a much lower position along the cell columns because of proliferative-zone enlargement. However, after treatment with 17-DMAG, the patterns of AP activity in TD and vit D- chicks were very similar to those observed in the normal growth plate. Parathyroid hormone-related peptide (PTHrP) is a key regulator of growth-plate development (20), and binding to its receptor
(PTH/PTHrPR) results in stimulation of chondrocyte proliferation and delay of hypertrophic differentiation in the lower proliferating zone, where cells are maintained in the pre-hypertrophic phenotype. In TD there are no changes in PTH/PTHrPR gene expression, and in rickets down-regulation of PTH/PTHrPR gene expression occurs probably because of the high PTH levels (3). Treatment with 17-DMAG did not affect PTH/PTHrPR in TD but prevented its down-regulation in rickets. No effect of 17-DMAG on cell differentiation, vascularization, or growth-plate size was observed when it was administered to the control chicks (data not shown).

**Hsp90 and hypoxia**

No hypoxia was observed in the control chicks, and HIF-1α was expressed by chondrocytes of the hypertrophic zone. A major increase in hypoxia was observed in the hypertrophic zone of TD growth-plate together with an increase in the expression of the HIF-1α gene that was especially evident above and below the lesion. In rickets, minor hypoxia was observed only adjacent to blood vessels (Fig. 3), and HIF-1α was expressed by cells of the lower hypertrophic zone at a lower location along the chondrocyte columns. Treatment with 17-DMAG resulted in elimination of hypoxia in both disorders, and the HIF-1α returned to the control levels.

Addition of 17-AAG, which is a geldanamycine analog and a specific inhibitor of Hsp90 activity to primary cultures of avian growth-plate chondrocytes was associated with decreases in the gene expression of HIF-1α and also of glucose transporter-1 (Glut-1), a downstream gene product of HIF-1α that regulates glucose uptake (Fig.4).

**Hsp90 and angiogenesis**

No changes with respect to the control were observed in the levels of VEGF in the TD- or rickets-afflicted chicks as a result of treatment with 17-DMAG (Fig. 5A). On the other hand, a significant reduction in the levels of Flk-1 that were observed in both bone disorders was restored after 17-DMAG treatment. Image analysis of the Flk-1 signal revealed a significant reduction ($P < 0.05$) in the TD and rickets Flk-1 compared with the control (arbitrary units - control 29,125±5,000; TD 17,369±3,300; vit D- 10,205±2,600) and increase back to control levels after 17 DMAG treatment (TD 38,444±5,100 and vit D- 22,387±983). As a result, the unvascularized growth
plates of TD and vitamin D-deficient chicks were invaded massively by blood vessels and became indistinguishable from the controls (Fig. 5B).

**Inhibition of Hsp90 and established TD**

To evaluate the effect of Hsp90 inhibition on chicks with established lesions, chicks were treated for 14 days with thiram starting on day 3 (Fig. 6). On day 7 chicks (n = 5) were sacrificed and TD lesions were scored. At this stage all chicks exhibited TD lesions (data not shown). The remaining chicks were divided into two groups, one of which was treated with 17-DMAG on days 7, 10 and 12. On day 14, the thiram-treated chicks exhibited enlarged growth plates (3.67±0.3 mm) and could barely stand or walk, whereas the size of the growth plates of chicks treated with 17-DMAG did not differ from those of the control untreated chicks (0.95±0.1 mm compare to 0.88±0.2 mm in the control chicks), and the treated chicks exhibited no signs of lameness.

**DISCUSSION**

The avian growth plate is populated by long columns of chondrocytes, and it requires a much higher level of vascularization than the mammalian one in order to provide the tissue with the necessary oxygenation. As a result, the avian growth plate is not hypoxic (Fig. 3) whereas hypoxia is observed in the mammalian one (31). In the growth plates of the long bones there is a close and dynamic interaction between developing vascular structures and cartilage maturation. In mice, vascular invasion of cartilage is associated with chondrocyte apoptosis, and inhibition of angiogenesis delays chondrocyte cell death and results in enlarged growth plates, with a massive expansion in the number of hypertrophic chondrocytes (47). Unvascularization and enlarged growth plates are the characteristics of avian TD and rickets (Table 1, Figs. 1, 5). TD and rickets are of dissimilar etiologies in that: A- levels of plasma calcium, phosphorus and 25(OH)D₃ levels and bone ash are normal in TD, but low in rickets (Table 1); B- the PTH/PTHrP axis is not involved in TD whereas in rickets down-regulation of the PTH/PTHrP receptor is observed, probably because of high PTH levels (Fig. 2); C- the TD lesion is hypoxic, which results in a major increase in HIF-1α gene expression, whereas in rickets only a minor increase in hypoxia is observed, without any changes in HIF-1α expression (Fig. 3); On the other hand, both disorders
elicit lameness (Fig. 1), abnormal differentiation of chondrocytes (Fig. 2), and decreases in Flk-1 levels (Fig. 5A).

Hsp90 is involved in regulation of protein folding and translocation of proteins across membranes, and it is an important mediator of cell growth, differentiation, survival and angiogenesis (35). Among its clients are pro-angiogenic regulator proteins such as HIF-1α, VEGF, and VEGF receptors. The growth-plate chondrocytes adjacent to the blood-vessels front exhibit HIF-1α (Fig. 4), and VEGF and Flk-1 (Fig. 5). In the normal growth plate HIF-1α is expressed in a hypoxia-independent manner mainly in the hypertrophic zone, where it probably is required for chondrocyte maturation and differentiation (28). In TD the growth plate becomes hypoxic and an additional wave of HIF-1α appears, mainly below the TD lesion. The Flk-1 levels are low, both in the rickets growth plate with minor hypoxia where there is no additional increase in HIF-1α expression, and in the hypoxic growth plate of the TD-afflicted chicks, where there is high HIF-1α expression, all of which suggest that Flk-1 expression is not regulated by hypoxia and HIF-1α.

Because of simultaneous involvement of Hsp90 in multiple steps of angiogenesis, various inhibitors were developed for combating tumor development (21,23,29). In contrast to the anti-angiogenic effect of Hsp90 inhibitors, observed in various tumors and other mammalian tissues (35), increased blood-vessel formation was observed in TD and rickets (Fig. 5), in which the low level of Flk-1 (Fig 5) is one of the rate-limiting factors of blood vessel formation that resulted in the unvascularized growth plate. Whereas in tumors inhibition of Hsp90 activity resulted in decrease in the VEGF/VEGF receptor axis, in TD and vit D- chicks increases in Flk-1 levels were observed that were associated with major increases in vascularization (Fig. 5). Once vascularization was restored, the growth plate became normoxic and the HIF-1α level decreased in vivo (Fig. 4) and in growth-plate chondrocytes in culture inhibition of Hsp90 resulted in inhibition of the expression of HIF-1α and its downstream Glut-1 gene (Fig. 4) that is essential for survival of the chondrocytes in the hypoxic environment (8,32). In addition, normal differentiation of chondrocytes was re-established, the size of the growth plate returned to normal, and the lameness disappeared. Moreover, inhibition of Hsp90 was effective not only in preventing thiram-induced TD and vitamin D-deficiency rickets, but also in reducing growth-plate size and eliminating lameness in chicks with established TD lesions (Fig. 6). It is important to note that no change in histopathology or in vascularization was observed
in the growth plate of the control chicks after 17-DMAG treatment, probably because of initially high levels of Flk-1 and maximal blood-vessel occupancy of the normal growth plate.

The improvement in the growth-plate histopathology and the amelioration of lameness in rickets, without any changes in plasma calcium, phosphorus, 25(OH)D₃ and bone ash, suggest that the effect of 17-DMAG was a local one and did not involve improvement in intestinal absorption or renal re-absorption of minerals, or any changes in the vitamin D status. Thus, it can be speculated that the observed improvement was temporary and that continued exposure of chicks to thiram or vit D-diets would restore the disorder. As in tumors (41), increase in Hsp90 was observed in the unvascularized growth plate of the TD-afflicted chicks (10), whereas no such increase was observed in the vitamin D-deficient growth plates (data not shown). The changes in growth-plate histopathology observed in rickets after 17-DMAG are in agreement with the notion that 17-DMAG affect Hsp90 activity rather than its synthesis.

The inhibitor dosage and Hsp90 occupancy may be among the reasons for the disparity observed between the angiogenic responses of tumors and of avian growth plates to Hsp90 inhibition. The effects of 17-DMAG and other Hsp90 inhibitors on HIF-1α and downstream target genes were dose-dependent: low doses of Hsp90 inhibitors increased and high doses reduced hypoxic HIF-1α protein levels. As a result the levels of downstream targets of HIF-1α, such as carbonic anhydrase IX, prolyl-4-hydroxylase domain protein 3, and VEGF, were affected accordingly (16). Moreover, in the chick chorioallantoic membrane angiogenesis assay, low doses were pro-angiogenic whereas high doses resulted in impaired and decreased blood-vessel formation. Although the doses used in the present study were compatible with those used in mammals, levels of Hsp90 occupancy and responses of client proteins may vary. Sensitive Hsp90 client-protein levels directly mirror Hsp90 occupancy at all time points, whereas for insensitive client proteins, protein abundance matches Hsp90 occupancy only after prolonged incubation with the drug (40). We cannot rule out the possibility that doses of 17-DMAG other than those used in this study may cause different effects in TD and rickets- afflicted chicks. The list of Hsp90 client proteins comprises many kinases, transcription factors, and other molecules known to be involved in cell proliferation, differentiation, apoptosis, survival and migration (48), therefore it cannot be ruled out that inhibition of Hsp90 affected other, as yet
unknown client proteins involved in vascularization, differentiation, and survival of chondrocytes within the growth plate.

PERSPECTIVE AND SIGNIFICANCE

This is the first report of the involvement of Hsp90 in chondrocyte differentiation and vascularization of the avian growth plate. In contrast to the anti-angiogenic effect of Hsp90 inhibitors observed in tumors and other mammalian tissues, inhibition of Hsp90 activity in the unvascularized TD and rickets-affected chicks resulted in increased Flk-1 levels, activation of the angiogenic switch, and restoration of normal growth-plate morphology.

ACKNOWLEDGMENTS

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DISCLOSURES

All authors have no conflicts of interest.
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Figure Legends

**Fig. 1.** – Inhibition of Hsp90 activity, growth-plate size and lameness. At the end of the experiment the chicks were photographed, the tibia growth plates were removed, photographed, stained with H&E and their size marked. Note the lameness and the enlarged growth plate in the chicks with TD and rickets before treatment and the recovery from lameness, and the normal growth plate size after 17-DMAG treatment. AC- articular cartilage; GP- growth plate; TDL- TD lesion.

**Fig. 2** *In situ* hybridization for collagen type II (Col II) and PTH/PTHrP receptor (PTH/PTHrPR) and staining for AP activity was performed on growth plates from chicks afflicted with TD and rickets, before and after 17-DMAG treatment. Col II magnification ×100; PTH/PTHrPR magnification ×40 insert ×5; AP magnification ×5. TDL- TD lesion, AC- articular cartilage, PZ- proliferative zone, TZ- transition zone, HZ-hypertrophic zone, BV- blood vessels, GP- growth plate

**Fig. 3.** - Effects of inhibition of Hsp90 activity on growth-plate hypoxia and Hif-1α levels were evaluated *in situ* by injecting chicks with Hypoxyprobe-1 at 40 mg/kg. After 60 min they were sacrificed and the tibiae growth plates were immunostained with antibodies that recognized Hypoxyprobe adducts (arrows). Hif-1α was detected by immunostaining. PZ- proliferating zone; HZ- hypertrophic zone; BV- blood vessels; TDL- TD lesion. Magnification ×100

**Fig. 4** - Effects of inhibition of Hsp90 activity in primary growth-plate chondrocytes on Hif-1α and Glut-1 gene expression. Hif-1α and Glut-1 gene expression was evaluate after incubation for 3 days with 17-AAG. The results are expressed in arbitrary units as the mean ± SE of 5 replicated experiments.

**Fig. 5.** - Effects of inhibition of Hsp90 activity on vascularization. A- VEGF and Flk-1 were evaluated by immunohistochemistry and the relative levels were calculated by image analysis. The results are presented as arbitrary units of the mean of 8 sections from each growth plate from 3 different chicks from each group ± SE. B- Blood vessels were evaluated by αSMA antibodies. TDL- TD lesion. Magnification ×5
Fig. 6. To evaluate the effect of inhibition of Hsp90 activity on established TD lesions the chicks were photographed on day 14 and the tibia growth plates (GP) were removed, photographed and stained with H&E. Note the lameness and the enlarged growth plate in the TD chicks before treatment and the recovery from lameness and the normal growth-plate size after 17-DMAG treatment.
Table 1 Effect of 17-DMAG on body weight, bone ash, growth plate length, plasma calcium, phosphorus and 25(OH)D₃ in TD and vit D- afflicted chicks.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>BW (g)</th>
<th>Bone ash (%)</th>
<th>GP length (mm)</th>
<th>Plasma Ca (mg/dL)</th>
<th>Ionic Ca (mmole/L)</th>
<th>Plasma P (mg/dL)</th>
<th>25(OH)₃ (ng/mL)</th>
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<tr>
<td>Cont</td>
<td>271±6a</td>
<td>33.8±0.6a</td>
<td>0.76±0.2a</td>
<td>11.1±0.4a</td>
<td>1.33±0.06a</td>
<td>6.1±0.8a</td>
<td>63.1±4a</td>
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<td>TD</td>
<td>165±7b</td>
<td>31.1±0.8a</td>
<td>2.76±0.7b</td>
<td>12.1±0.6a</td>
<td>1.40±1.40a</td>
<td>7.2±0.9a</td>
<td>62.1±3a</td>
</tr>
<tr>
<td>TD + 17DMAG</td>
<td>203±10c</td>
<td>32.1±0.7a</td>
<td>0.90±0.2a</td>
<td>11.5±0.6a</td>
<td>1.39±0.05a</td>
<td>6.3±0.8a</td>
<td>63.5±4a</td>
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<tr>
<td>Cont</td>
<td>305±12a</td>
<td>38.4±1a</td>
<td>1.28±0.3a</td>
<td>11.2±0.2a</td>
<td>1.25±0.02a</td>
<td>6.9±0.2a</td>
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<td>Vit D-</td>
<td>268±12b</td>
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<tr>
<td>Vit D- +</td>
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<td>0.86±0.06b</td>
<td>5.4±0.4b</td>
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Analysis of body weight (BW), bone ash, growth plate (GP) length, plasma total and ionic calcium, plasma phosphorus (P) and plasma 25(OH)D₃ in control, TD and vit D-chicks, with and without the Hsp90 inhibitor 17-DMAG. The results are the mean ± SE of 8 chicks. Means in the same column and in the same experiment with no common superscript differ significantly (P < 0.05).
Cont | TD-prevention | TD-treatment | Vit D-

| -DMAG | +DMAG | -DMAG | +DMAG | -DMAG | +DMAG |

Control diet | Thiram 40ppm | Thiram 25ppm | Vitamin D-deficient diet

| 3d | 4d | 5d | 10d | 3d | 7d | 10d | 12d | 14d | 1d | 3d | 7d | 12d |

Saline/DMAG 600μg
Hypoxia

Hif-1α

Cont

TD

Vit D-

-DMAG

+DMAG

-DMAG

+DMAG

PZ

HZ

PZ

HZ

TDL

BV

BV

BV