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Review

PREECLAMPSIA: CARDIOTONIC STEROIDS, FIBROSIS AND A HINT TO CANCEROGENESIS

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Abstract: Despite prophylaxis and attempts to select a therapy, the frequency of preeclampsia does not decrease, and it still takes the leading position in the structure of maternal mortality and morbidity worldwide. In this review, we present a new theory of the etiology and pathogenesis of preeclampsia which is based on the interaction of Na/K-ATPase and its endogenous ligands including marinobufagenin. The signaling pathway of marinobufagenin involves an inhibition of transcriptional factor Fli1, a negative regulator of collagen synthesis, followed by deposition of collagen in the vascular tissues and altered vascular functions. Moreover, in vitro and in vivo neutralization of marinobufagenin is associated with restoration of Fli1. The inverse relationship between marinobufagenin and Fli1 opens new possibilities in the treatment of cancer: since Fli1 is a proto-oncogene, a hypothesis on suppression of Fli1 by cardiotonic steroids as potential anti-tumor therapeutic strategy is discussed as well. We propose a novel therapy of preeclampsia which is based on immunoneutralization of the marinobufagenin by monoclonal antibodies, which is capable to impair marinobufagenin-Na/K-ATPase interactions.

Keywords: preeclampsia; Na/K-ATPase; marinobufagenin; Fli1; TFG-beta; collagen-1; vascular fibrosis

1. Introduction.

Preeclampsia (PE) is one of the most common hypertensive disorders developing during pregnancy [1]. Affecting approximately 2-8 % of pregnancies worldwide, it increases the risk of complications for both mothers and babies [2]. Despite the ongoing prophylaxis and repeated attempts to select a therapy, it is still not possible to reduce the incidence of PE. As a result, it is one of the leading causes of maternal and perinatal morbidity and mortality. The major clinical manifestation of PE is increased blood pressure (≥ 140/90 mm Hg registered in at least two measurements with 6-hours interval) after 20 weeks gestation [1], however, the definite diagnosis of PE requires a combination of such features as hypertension, proteinuria (the presence of ≥ 300 mg of protein / L of urine in a daily sample or in two samples taken 6 hours apart), generalized edema, and any associated organ dysfunction [2,3].

Despite many investigations, the etiology of PE is not completely understood. The pathophysiological mechanisms of PE revealed to date include placental abnormalities or injury,

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general endothelial dysfunction, abnormal production of angiogenic factors and vasoactive substances, placental steroid and peptide hormones, oxidative stress, insulin resistance, disturbed immune interaction between maternal organism and placenta [4, 5]. The final result of this complex interaction is a multisystem disorder characterized by hypertension, proteinuria and multiple end-organ ischemia or dysfunction. The present review is focused on the mechanisms related to abnormal processes in blood vessels and presents the "fibrotic" concept of PE development. This model postulates that one of the major causes of PE is linked with enhanced synthesis of cardiotonic steroids (CS) which leads to disturbances in intracellular signaling and finally to altered vascular reactivity including arterial fibrosis. Immunoneutralization of CS as a valuable approach to relieve the symptoms of PE and a promising therapy is discussed as well.

2. Bufadienolide cardiotonic steroids

The search for candidates for the role of natriuretic hormones has found several circulating steroid compounds represented by cardenolides which include ouabain and digoxin, and bufadienolides such as marinobufagenin [6]. Bufadienolide CS differ from cardenolides by the presence of a twice-unsaturated six-membered lactone ring. For several decades, it was known that some representatives of the amphibians, for example, the toad Bufo marinus, can synthesize the steroids of bufadienolide group [7]. The endogenous level of bufadienolides in toads increases following their migration from arid habitats to areas with high humidity [7]. Since amphibian skin is the most important organ involved in the regulation of water-salt balance, it has been suggested that the sodium pump and bufadienolides are physiological regulators of sodium transport [8]. Soon, a hormone with mass spectral characteristics identical to one of amphibian bufadienolides - marinobufagenin (MBG) was isolated from the urine of patients with myocardial infarction [9]. In the in-vitro and in-vivo experiments, monoclonal antibodies to MBG restored the activity of Na/K-ATPase [10]. Later it was shown that MBG is synthesized by the cells of adrenal cortex and placenta by transformation of bile acids using one of the enzymes of the P450 cytochrome family – CYP27A1 [11]. MBG selectively interacts with the alpha-1 isoform of Na/K-ATPase, the main isoform of the enzyme in the kidneys and blood vessels, and it is an active vasoconstrictor and natriuretic [6,12]. The content of MBG in blood plasma increases with the rise in volume of circulating fluid and with sodium retention, for example, in patients with hypertension and chronic renal failure, as well as with congestive circulatory failure [13,14].

3. Cardiotonic steroids, pregnancy and preeclampsia

Since a normal pregnancy is accompanied by fluid retention and a positive sodium balance in the body [15], interest in the possible role of CS in pregnancy arose shortly after their discovery. Graves et al. showed that the level of digoxin-like immunoreactivity increases moderately in a healthy pregnancy and rises significantly with gestational hypertension [16]. Based on these observations, the authors suggested that excessive CS synthesis may be one of the factors implicated in PE pathogenesis [16]. Over the next few years, the findings of Graves et al. found their confirmation in the works of other clinicians [17,18,19,20]. Convincing evidence of the contribution of CS to the pathogenesis of PE was obtained in experimental and clinical studies using the Digibind preparation, which is a lyophilized Fab fragment of affinity-purified sheep anti-digoxin antibodies and has cross- immunoreactivity with several CS [21,22]. The administration of Digibind caused a decrease in blood pressure in rats with volume-dependent hypertension, accompanied by an increase in the concentration of CS in blood plasma [23]. During pregnancy, the site of synthesis of CS is the placenta [24]. Di Grande et al. showed that Digibind at a concentration of 130 μg / ml causes a significant decline in vascular tone of perfused placentas obtained after delivery from patients with PE [25]. Armler et al. found that the development of PE is accompanied by a significant increase in the sensitivity of placental Na/K-ATPase to digitalis preparations [26]. Moreover, it is the cells of placental origin, JEG-3, which were found to synthesize MBG from the bile acids using the enzyme CYP27A1 [11].

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Clinical studies suggest that one of the mechanisms of PE pathogenesis is excessive production of MBG. Thus, MBG content in the plasma of pregnant patients with severe PE (blood pressure 161/104 mmHg) was enhanced by 5 times compared to that in women with uncomplicated pregnancy, while in the case of moderate PE (blood pressure 149/93 mmHg) - by two times [27,28]. Later, it was shown that such two-fold increase in the level of MBG in blood plasma following development of moderate PE was accompanied by a 50% inhibition of the activity of Na/K-ATPase in red blood cells, whereas antibodies to MBG, unlike antibodies to ouabain, restored the activity of enzyme activity ex vivo [28]. These observations indicate that MBG is a factor "responsible" for the suppression of Na/K-ATPase activity in PE, as well as a marker of the severity of this syndrome. In in vitro experiments, MBG in the "pathophysiologically significant" concentration range (1-3 nmol/L) induced 25% inhibition of Na/K-ATPase in human sarcolemma and triggered a contractile reaction in isolated rings of the human mesenteric arteries [27,28]. Therefore, MBG levels observed in patients with PE in vivo can increase vascular tone and significantly inhibit Na/K-ATPase [28].

4. Fibrosis and preeclampsia

During PE, the spiral arteries of placenta lose their elastic properties, which ultimately leads to poor placental perfusion [29,30]. This phenomenon was established to be associated with another important effect of CS, their ability to function as pro-fibrotic factors. Recent studies have shown that, in addition to vasoconstrictor effect, CS are important regulators of intracellular signaling cascades, the violation of which leads to loss of elasticity and vascular fibrosis [31,32]. Both effects of CSs, interacting with each other, are associated with remodeling of myocardial tissue and blood vessels, along with other processes leading to the deposition of collagen and impaired ability of vessels to relax [33]. Recently, it was shown that nanomolar concentrations of MBG stimulate collagen synthesis and induce fibrosis in the cardiovascular and kidney tissues [33,34]. Culture of the fibroblast with CS was shown to enhance the collagen synthesis in cells, which confirms the signaling function of the MBG-Na/K-ATPase complex, different from the classical function of the sodium pump (Figure 1) [32]. In vivo administration of MBG to rats at a concentration observed in the blood plasma of patients with renal failure caused myocardial fibrosis, accompanied by activation of the Na/K-ATPase-mediated signaling pathway, which was confirmed by the increased expression of Src kinase and phosphorylation of one of the mitogen-activated protein kinases (MAPK) - ERK1/2 in the myocardium [32]. One of the most important mechanisms underlying the pro-fibrotic effect of MBG is the altered activity of Fli1, a nuclear transcription factor, and a negative regulator of collagen-1 synthesis [33,34]. Inhibition of Fli1, a nuclear transcription factor, and a member of the ETS family, is critical for MBG-induced fibrosis [32]. Fli1 acts as a negative regulator of collagen-1 synthesis and it competes with another transcription factor, ETS-1, to maintain a balance between stimulation and repression of the collagen-1 gene [35,36]. The Na/K-ATPase/Src/EGFR complex begins a signal cascade, which activates phospholipase C (PLC) resulting in phosphorylation of PKCδ and its translocation to the nucleus. In the nucleus, phosphorylated PKCδ phosphorylates Fli1, which withdraws Fli1-induced inhibition of the collagen-1 promoter and increases in procollagen expression and collagen production [32] (Figure 1). The mechanism of vascular fibrosis induced by various vascular factors including CS is also implicated in remodeling of spiral uterine arteries, associated with the development of PE [32,37]. Interestingly, the same mechanism of synthesis of collagen-1, Fli1-dependent fibrosis, was found in myocardium of rats with renal failure [38].

In our study performed on umbilical arteries obtained after delivery from patients suffering from PE, the collagen content in vessels was much higher, but the level of Fli1 was lower compared to those in the arteries of women with an uncomplicated pregnancy, and the arteries themselves were less sensitive to the relaxing effect of sodium nitroprusside [33,34]. The suppression of Fli1 and excessive synthesis of collagen-1 in the placenta and umbilical arteries obtained from the patients with PE was confirmed by another recent study [37]. Previously, it was also shown that the blood plasma of women with PE contains an increased amount of MBG [27,28]. In pregnant rats, an increase in MBG content caused by consumption of NaCl was accompanied by the development of

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typical symptoms of PE, including increased blood pressure, proteinuria, and decreased weight and size of fetuses [39]. Taking into account that MBG stimulates the synthesis of collagen, the development of fibrosis in placenta and umbilical arteries of patients with PE is accompanied by increased production of MBG and substantial suppression of Fli1 [36], and that in PE vascular stiffness is based on elevated MBG level [33,40], one can conclude that MBG is one of the major factors involved in the pathogenesis of PE through induction of vascular fibrosis. In addition, it is assumed that blood vessels exposed to the negative effects of PE factors are subsequently more sensitive to damage, despite the disappearance of symptoms of PE after the delivery [41]. It should be noted, that the Fli1-dependent mechanism is not a single mechanism of MBG-induced fibrosis. In several experimental models, including salt-loaded young Sprague-Dawley rats and salt-sensitive Dahl rats MBG exerts its pro-fibrotic effect acting via activation of TGFβ and SMAD2-3 signaling pathway and activation collagens-1,-2,-3,-4 and -5 [42,43]. Interestingly, in experimental salt-loaded rats with type-2 diabetes mellitus both mechanisms of fibrosis in aorta and myocardium, Fli1- and TGFβ-dependent, were taking place [44].

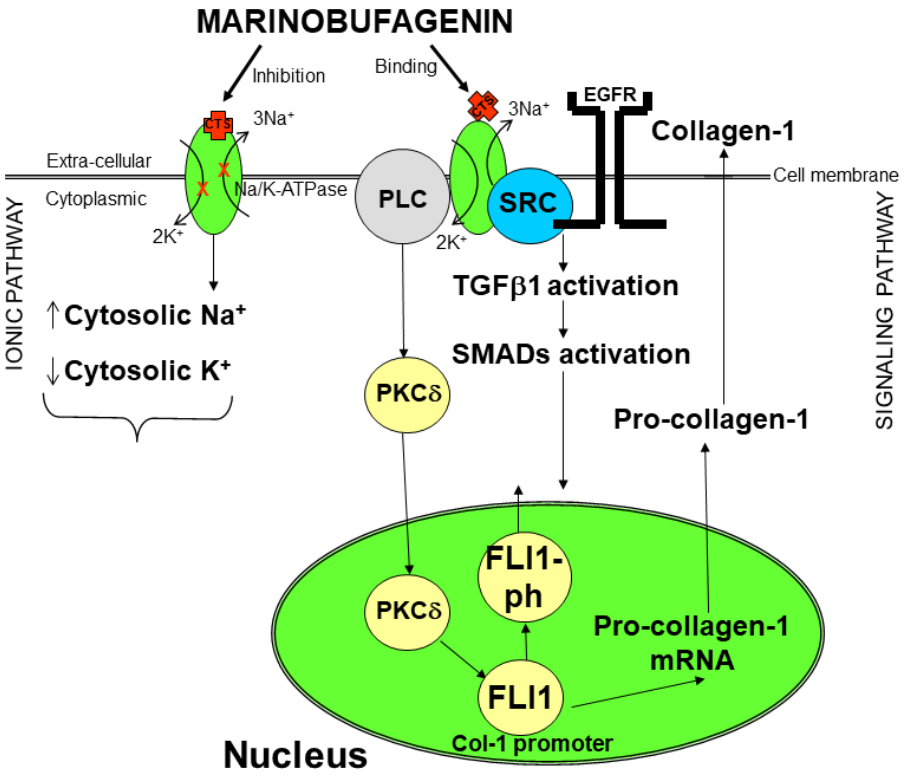

Figure 1. Schema of "classical" vs. "signaling" pathways for CS effects. In the classical pathway, any signaling through the Na/K-ATPase requires the inhibition of the Na/K-ATPase pumping activity which, in turn, is accompanied by changes in cytosolic Na^+ and K^+. The increases in [Na] and decreases in [K] induce increases in cytosolic [Ca] which, in turn, activates a variety of pathways which involve combinations of genomic and non-genomic effects. The "signaling" pathway involves the association of Src with the Na/K-ATPase. Binding of the CS to the Na/K-ATPase activates Src which transactivates the epidermal growth factor receptor (EGFR) and phospholipase C (PLC). This leads to generation of cascades which involve generation PKCδ and activation of Fli1, or activation of TGFβ and SMAD, and, finally activation of collagen-1 and fibrosis.

5. Immunoneutralization of cardiotonic steroids

The idea of immunoneutralization of CS in patients with PE is not new. The similarity of the structure of cardenolides (digoxin, ouabain) and bufadienolides (MBG) allowed the use of

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polyclonal antibodies against digoxin (Digibind and Digifab) for the treatment of PE in clinical practice [45,46]. Digibind has been used for many years to relieve the severity of symptoms in patients after poisoning with digoxin [47]. Over the past 20 years, digoxin-specific Fab fragments (Digibind) have been successfully used to treat intoxication with bufadienolides of the toads and be effective in animals and humans [48,49]. Administration of Digibind was shown to decrease blood pressure in animals with experimental hypertension due to interaction with an endogenous digoxin-like factor [50]. In 1988, Goodlin achieved a persistent decrease in blood pressure in a pregnant woman with PE which developed at 26 weeks of gestation after twice intravenous administration of Digibind at a dose of 0.087 mg/kg [19]. Graves and co-authors, using Digibind-based immunoassay, demonstrated the placental origin of human CS and showed that ketoconazole, a steroid synthesis inhibitor, inhibited its biosynthesis [51]. Importantly, placental hypoxia was shown to increase placental CS release [51]. Adair et al. observed a decrease in blood pressure in a patient with PE on the background of combined intravenous bolus of 5 mg Digibind and a 24-hour infusion of the drug (1 mg per hour) [52]. Somewhat later, the effectiveness of Digibind was confirmed in a double-blind, placebo-controlled study in 13 patients with PE developed *post partum* [53]. Another positive finding was that in patients with PE treatment with Digibind resulted in an increase of creatinine clearance vs. that in the placebo group [54]. Multicenter study of effectiveness of Digibind for the treatment of pregnant women with severe PE, completed in 2007, has shown that the administration of Digibind leads to a significant increase in creatinine clearance and decreases the risk of pulmonary edema compared with the patients who did not receive antibodies [55]. It is noteworthy that in the above studies Digibind did not cause any side effects in patients. However, in 2011 the production of Digibind was discontinued, and Digifab (BTG International Ltd., United Kingdom) was the only digoxin antibody preparation registered with the US Food and Drug Administration. A comparative study of Digifab and Digibind showed that they have comparable cross-reactivity with bufadienolides and cardenolides [56]. In patients with PE, an increase in immunoreactivity to MBG was identical for both Digifab and Digibind, and both antibody preparations restored Na/K-ATPase activity [56].

6. Interaction of CS and Fli1, and a hint for cancer.

As mentioned previously, when antibodies to CS are administered to experimental animals or are applied to tissues from pregnant humans concentrations of Fli1 in exhibit increases [33,34]. Levels of MBG in plasma are in a reciprocal relationship with the level of Fli1, a member of the ETS family, and the anti-fibrotic factor [32]. When levels of MBG are increased, levels of Fli1 decrease, and Col-1 gene promoter is released from the nucleus, and pro-collagen-1 and collagen become activated [32]. While many studies demonstrate that Fli1 is a pro-cancer factor [57,58], CS, including MBG, are becoming attractive anticancer drugs [59,60]. It is generally accepted that PE is associated with a low risk of cancer, which is not surprising considering that endogenous levels of MBG and other CS are dramatically increased in PE patients [27,28] and therefore suppress the growth of tumors in vivo and in vitro [61,62,63]. Therefore, theoretically, in cancer, patients and experimental animals, MBG should suppress levels of Fli1, an oncogene. This notion is an agreement with the data showing the levels of Na/K-ATPase inhibitors were measured in breast cancer patients. In that study, Weidemann found that a majority (73.6%) of 84 expressed lower CS plasma concentrations (<50 pmol/L) than that in the control group (150 pmol/L)[64,65]. This observation has been confirmed quite recently, and when levels of endogenous bufalin in the serum of patients with hepatocellular carcinoma were compared with controls they were 4 times lover [66]. Interestingly, when levels of CS were compared in the nude mice expressed significantly lower CS concentrations in the adrenal gland and the plasma compared to normal mice [67]. The nude mouse is valuable to research because it can receive many different types of tissue and tumor grafts, as it mounts no rejection response, and it has a very high prognostic value of FLI1 [68,69].

Fli1 belongs to the Erythroblast Transformation Specific (ETS) family, one of the large family of transcription factors that are highly conserved and are unique to animals [70,71]. The ETS family is involved in a wide variety of functions including the regulation of cellular differentiation,

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control, cell proliferation, apoptosis, angiogenesis, and are associated with cancer [71]. The Fli1 is encoded by *the FLI1* gene, which is a proto-oncogene, Fli1 was first identified in cancer, systemic sclerosis, and tissue fibrosis [72,73]. This phenotype was consistent with a role of Fli1 as a regulator of vessel maturation, thus, in rats following subtotal nephrectomy, elevated MBG led to a reduction in the level of Fli1 and an increase in collagen-1 level in the myocardium, and single administration of monoclonal anti-MBG antibody rats produced an antifibrotic effect, that is, restored Fli1 levels and reduced collagen-1 abundance in the myocardium [38]. Fli1 attracted attention primarily because of its contribution to different types of cancer including, gastric cancer, Burkitt lymphoma, breast cancer, pancreatic ductal adenocarcinoma, small cell lung cancer, and Ewings sarcoma [57,74,75,76]. We observed extremely high levels of MBG, low levels of Fli1, along with a very extremely high level of collagen-1 level in patients and experimental animals with preeclampsia, chronic renal failure, and malignant hypertension [33,37,38]. When animals from all three groups were given 3E9 monoclonal antibody against MBG it was associated with an increase in Fli1 and dramatic reduction of fibrosis, suggesting that CS are potentially anti-cancer substances [33,37]. This line of evidence agrees with the results of a study was conducted with the participation of 9271 patients, which showed an association between a high concentration of digitoxin in blood plasma and a low risk of developing malignant neoplasms of the blood and hematopoietic organs, as well as a moderate decrease in the incidence of kidney cancer, urinary tract cancer and prostate cancer [77].

These retrospective observations are largely confirmed by in vitro studies, indicating the possibility of a direct inhibitory effect of CS on the proliferative and metabolic potential of various types of tumor cells [78]. For example, increased (compared to other tumors) expression of the α1-Na/K-ATPase subunit is observed in non-small cell lung cancer, renal cell carcinoma, gliomas, and melanoma, and an increase in the α3-Na/K-ATPase subunit is observed in colon cancer [80,81,82,83,84]. Several authors note a decrease in the content of the α1-Na/K-ATPase subunit is observed in prostate cancer [85], while Kiss et al. suggested that α1 subunit is a new target especially in the therapy Glioblastoma [79]. It is necessary to highlight that there is a significant increase in the intracellular concentration of Na+ and, an increase in the content of Ca2+ in cells, along with a moderate decrease in the intracellular concentration of K+[81]. Effect of CS differ depending on dose, thus Li et al demonstrated that in a human gastric cancer cell line (MGC803), bufalin at 20 nmol/L induced M-phase cell cycle arrest, whereas at 80 nmol/L, it induced apoptosis via an increased Bax/Bcl-2 ratio and activated caspase-3 [86]. These distinct effects were correlated to transient activation of the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway [86]. Proscillaridin A was identified as a potential treatment compound with IC_{50} values ranging from 0.007 μM to 0.55 μM in various tumor types [87]. Importantly, the number of studies in which bufadienolides were used as an in-vitro anti-cancer treatment is heightened, and bufadienolide inhibitors of the Na/K-ATPase are used in vitro and in vivo include MBG [59,60], bufalin [88,89], cinobufagin [90], resibufagenin [91], proscillaridin A [92], gamabufotalin [93], and 1α,2α-Epoxyscillirosidine [94]. Analyzing experiments and clinical data it is obvious that MBG and other Na/K-ATPase inhibitors hold promise to treat cancer, and following anti-CS antibody treatment to PE patients we must expect a rise of Fli1 and be alert. The direct link between cancerogenesis, MBG, and the activity of Fli1 is yet to be established.

7. Conclusions

Our studies confirmed the ability of antibodies to MBG to prevent the processes associated with PE. Thus, the introduction of antibodies to MBG ex vivo eliminated the inhibition of Na/K-ATPase in red blood cells obtained from the blood of patients with PE [27,28]. In the pregnant rats with experimental PE induced by consumption of water with excessive NaCl amount, the in vivo immunoneutralization of MBG by poly- and monoclonal antibodies exerted an antihypertensive effect associated with the restoration of vascular Na/K-ATPase activity [10]. A recent work also showed that incubation of explants of umbilical arteries obtained from the patients with PE with monoclonal antibodies against MBG led to a significant decrease in collagen-1 content [37]. Besides, incubation of healthy human umbilical arteries in the presence of low MBG concentrations for 24

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hours led to decline in the Fli1 content and an increase in PKC-delta expression, while the level of pro-collagen-1 synthesis increased six-fold [95]. Another work has shown that the introduction of humanized monoclonal antibodies to MBG leads to phosphorylation of MAP kinase p38 in cytotrophoblast cells, indicating a possible therapeutic effect of these antibodies [96]. Moreover, a recent study has demonstrated that silencing of Fli1 in human umbilical arteries mimics preeclamptic phenotype through activating PKCδ and activation of pro-collagen and collagen-1 synthesis [97]. We suggest that the interaction of MBG and Na/K-ATPase is the cornerstone in pathogenesis of PE. The development of PE is associated with increase in MBG production, which through the Fli1-dependent mechanism stimulates the synthesis of collagen in the umbilical arteries and finally leads to impairment of vasorelaxation and development of vascular stiffness which may progress beyond PE [33,37]. Understanding of the role of Fli1 and MBG in development of PE gives us a possibility to suggest CS as one of the therapeutic tools for the treatment of cancer. As measurement of MBG became available in pregnant plasma via sensitive analytical method relying on liquid chromatography combined to mass spectrometry [98], immunoneutralization of MBG may become an effective direction in the treatment of PE.

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/xxx/s1.

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Abbreviations

CS – cardiotonic steroids
EGRF - epidermal growth factor receptor
ERK – extracellular signal-regulated kinases
ETS - erythroblast transformation specific family
Fli1- Friend leukemia integration 1 transcription factor
MBG – marinobufagenin
SMAD - proteins, main signal transducers for receptors of the transforming growth factor beta
Src - a family of proto-oncogenic tyrosine kinases
PKC – protein kinase C
PLC - phospholipase C
PE – preeclampsia
TGFb - transforming growth factor beta 1

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