Heat shock proteins (HSPs) are chaperones with highly conservative primary structure, necessary in the processes of protein folding to the most energetically advantageous conformation and maintaining their stability. HSPs perform a number of important functions in various cellular processes and are capable of modulating pathophysiological conditions at the cellular and systemic levels. An example is the high level of HSP expression in neoplastic tissues, which disrupts the apoptosis of transformed cells and promotes the processes of proliferation, invasion, and metastasis. In addition, an increasing amount of information is appearing about the participation of HSPs in the formation of multidrug resistance. This paper provides a review of the current state of research on the fundamental importance as well as the diagnostic and prognostic role of various classes of HSP in cancer treatment. It presents the prospects for using HSPs as biological markers of disease progression and targets in various cancer treatment strategies. However, the need for additional research is quite high. Only numerous joint efforts of research groups will allow the effective use of HSPs as a tool to combat cancer.

**Key words:** cancer, heat shock proteins, apoptosis, anti-cancer therapy.

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**The role of heat shock proteins in neoplastic processes and the research on their importance in the diagnosis and treatment of cancer**

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**Introduction**

Heat shock proteins (HSPs) are an evolutionary old family of proteins with highly conservative primary structure (invariable regardless of organism). Their characteristic feature is the ability to interact with a large number of other proteins, which clearly distinguishes HSPs from most cellular proteins that usually interact with one or more molecular counterparts. HSPs take part in various cellular processes, such as protein folding into the most energetically advantageous conformation, transporting proteins through membranes, protecting and controlling their structure, processing antigens, or binding peptides to molecules of major histocompatibility complex class I [1, 2]. The main task of HSPs is to protect the cell by suppressing the effects of various stress factors, and therefore they are sometimes called chaperones. In a properly developing cell, HSPs constitute approx. 5–10% of the total amount of proteins. The transcription of HSP encoding genes and the increase in expression of these proteins is triggered by various environmental stress factors (e.g. high temperature, metabolic poisons, ionizing radiation) and in the course of pathophysiological processes in the body (viral and bacterial infections, as well as cancer). Because apoptosis and HSP expression are induced by the same factors, researchers have sought a correlation between these 2 processes. An extremely important role of HSPs is the ability to model the early stages of apoptosis, i.e. programmed cell death; therefore, in the last few decades these proteins have become the subject of extensive research as to their role in cancer. It has been demonstrated that excessive amounts of HSP are synthesized in tumour tissues, participating in the control processes of cell proliferation, differentiation, and programmed death, as well as contributing to the process of angiogenesis [3, 4]. Overexpression of these proteins can be observed in a number of neoplasms, such as prostate, bladder, breast, ovary, cervical, colon, lung, oesophageal, and kidney cancer [5]. All HSPs are divided into families according to the generally accepted classification depending on their molecular weight expressed in kilodaltons (kDa). Three HSP families play a significant role in neoplastic processes. They are HSP90, HSP70, and HSP27 with molecular weights of 90, 70, and 10–30 kDa (small HSP), respectively.

It has been demonstrated that the expression of HSP27 is a negative prognostic factor in gastric, prostate, and liver cancer, while HSP70 – in bladder, breast, and cervical cancer [6]. HSP90 expression is weakly correlated with breast cancer and is a favourable prognostic factor in endometrial can-
The role of HSP70 in apoptosis

The anti-apoptotic function of the HSP70 family is well understood. These proteins can act at many different stages of the signalling pathways. In vitro studies (U937 and Wehi-s lines) showed that KN apoptosis is directly dependent on the level of HSP70 synthesis. It has been observed that high expression of HSP70 prevents cell apoptosis induced by actinomycin D, camptothecin, and etoposide. The blocking of apoptosis is accomplished by high-molecular-weight HSPs (including HSP70) binding to caspases, which interferes with the activation of the latter. The neoplastic tissue accumulates a pool of cells with latent mutations, which promotes further tumour development [19].

HSP70 can block apoptosis at the premitochondrial, mitochondrial, and postmitochondrial levels. This takes place, among others, by interaction with the signalling pathway of the Fas (e.g. DR4 and DR5) and its tumour necrosis factor-related apoptosis-inducing ligand, interfering with the process of changing the mitochondrial membrane permeability and, finally, inhibiting apoptosis formation or protecting nuclear proteins against their cleavage by caspase-3 [20]. The anti-apoptotic effect of HSP70 is observed in cancer cells with either almost unchanged or significantly increased expression of the MYC oncogene. It is well known that the MYC transcription factor ensures a high level of cell proliferation (by increasing the activity of cyclin complexes and cyclin-dependent kinases of the G1 phase of the cell cycle – Cdk4 and Cdk2) and also that it can be associated with a high level of apoptosis (by increasing the expression of pro-apoptotic Bcl-2-associated X protein (BAX) protein). It has also been demonstrated that increased MYC expression makes neoplastic
cells more sensitive to the effect of anti-cancer drugs such as doxorubicin, etoposide, and camptothecin [21]. Under these conditions, increased HSP70 expression inhibits the development of apoptosis induced by these drugs. It appears that HSP70 interference with apoptotic signalling pathways associated with increased MYC expression may be the cause of acquired drug resistance of cancer cells.

**The role of HSP90 in apoptosis**

The HSP90 family is one of the most thoroughly studied members of HSPs showing anti-apoptotic properties. The molecular mechanisms of the anti-apoptotic action of HSP90 include a decrease in the activity of caspases-8 and -3, a decrease in the number of tumour necrosis factor and Fas receptors, a change in the level of transcription factors p53 and NF-κB, as well as an imbalance of pro- and anti-apoptotic proteins from the Bcl-2 family in favour of the latter. The predominance of the expression of anti-apoptotic proteins prevents the reduction of the mitochondrial transmembrane potential.

HSP90 and HSP70 are complementary. Thus, when cytotoxic drugs bind HSP90 there is an increase in HSP70 synthesis, which reflects the activation of the compensatory cellular protective mechanisms. As a consequence, there is an increased probability of survival of cancer cells and resistance to the above-mentioned drugs. Such a phenomenon has been described, for example, in medulloblastoma in children [22]. Blocking of HSP70 and its constitutive isoform, HSC70 (constitutive isoforms HSP), by siRNA causes proteosome-dependent degradation of HSP90 proteins in colon cancer tissues, resulting in extensive tumour apoptosis, as well as intensification of necrotic cell apoptosis with pharmacological inhibition of HSP90 [11].

**Involvement of heat shock proteins in the processes of metastasis**

HSPs have also been studied for their role in the processes of invasion and metastasis, which have a significant impact on overall survival. HSPs were first detected in cells. Subsequently, extracellular HSPs (ex-HSPs), membrane surface HSPs (mHSPs), and ev-HSPs contained in extracellular vesicles (oncosomes and exosomes) were also discovered [23]. Ex-HSPs play a key role in extracellular communication in the neoplastic process, in immune reactions, as well as in other processes, e.g. degenerative processes.

HSPs are released from cells passively, by cell damage or death, or actively by secretion of HSP-containing exosomes. Proteomics showed that oral cancer oncosomes are rich in HSP90, HSP70, HSC70, and HSP105 [23]. It is worth noting that HSP90α is highly expressed in neoplastic cells and secreted into the extracellular space in the form of free HSP90α and also as an oncosome load [24].

Ex-HSP, ev-HSP and mHSP can bind to cell surface receptors, stimulate intracellular signalling pathways, be absorbed by endocytosis, or transported molecularly to target cells such as immune cells, cancer cells as well as cancer stem cells, epithelial cells, cancer-associated fibroblasts, mesenchymal stem cells, tumour endothelial cells, and lymphoid endothelial cells [25].

Exosomes derived from primary tumour cells transfer oncogenic factors to cells in the tumour microenvironment and in the pre-metastatic niche [26]. Ex-HSP and ev-HSP play a key role in cancer development by binding to cell surface receptors such as CD91, promoting epithelial-mesenchymal transition (EMT), migration, invasion, heterogeneity, metastasis, drug resistance of cancer cells, and angiogenesis [27–29].

It has been shown, among others, that high serum concentration of soluble HSP27 and phosphorylated HSP27 in colorectal cancer patients who had undergone removal of lung metastases was associated with worse relapse-free and overall survival [30].

**Heat shock proteins as biological markers of neoplastic process**

Depending on the type of tumour and its stage of advancement, HSPs show differential expression; therefore, HSP testing may be of prognostic value.

The conducted studies have provided controversial results. The association between HSP expression and prognosis was demonstrated in colorectal cancer, whereas such a correlation was not found in hepatocellular carcinoma, [30, 31]. Decreased HSP27 expression was observed in the early stages of colorectal cancer (grade I and II), while in advanced stages (grade III and IV), increased HSP27 expression was demonstrated [32]. Intracellular HSP27 overexpression is observed in patients with prostate cancer [33]. The prognostic significance of HSP27 overexpression was found in patients with meningioma [34].

Given the ability of HSP to be released into the extracellular space, it has become appropriate to study ex-HSP as a potential cancer biomarker in body fluids using fluid biopsy.

In women with breast cancer, the levels of HSP27 in the serum and tumour microenvironment were significantly higher compared to the control group [35]. A high level of HSP27 was also demonstrated in the interstitial fluid isolated from the primary tumour tissue [36].

HSP70 is often found on the cancer cell plasma membrane and is released into the bloodstream through exosomes. Serum HSP70 levels were significantly higher in patients with lung cancer (both squamous and adenocarcinoma) than in the control group [37]. Serum HSP70 levels correlate significantly with total tumour volume in both types of cancer. The use of HSP70 as a tool for the early diagnosis of cervical cancer was demonstrated in both in vitro and in vivo studies [38]. HSP70 was overexpressed in patients in the first and second stage of the disease. Inhibition of HSP70 expression reduced the mobility of cancer cells and their invasive capacity. Increased expression of HSP70 was also demonstrated in the serum of patients with breast cancer, colorectal cancer, and acute myeloid leukaemia [39]. Moreover, longer survival times were observed in patients with high levels of anti-HSP antibodies.

Promising results were also obtained by studying extracellular and exosomal HSPs as diagnostic and prognostic biomarkers in patients with head and neck squamous cell carcinoma (HNSCC) [23] and prostate cancer [24].
HSP-rich exosomes were secreted by HNSCC cells with high metastatic capacity. They contained a significantly higher amount of tumour necrosis factor receptor-associated protein 1 (TRAP1), Hsp90β, Hsp90α, Hsp105/HspH1, and Hsp72 compared to HNSCC with low metastatic potential. Patients with tumours demonstrating high levels of TRAP1 or Hsp90β are characterised by an unfavourable prognosis. Patients with stage I and II HNSCC showed high expression of TRAP1 and Hsp105, while patients with stage III and IV had increased expression of Hsp90α/β. Studies on 3-dimensional models of PC-3 castration-resistant prostate cancer cell lines showed a significantly higher secretion of ex-HSP90α by hypoxic cells compared to smaller tumours and 2-dimensional cultures in which cells are much better oxygenated [24]. In this model, ex-HSP90α was released in large quantities, while ev-HSP90α was practically undetectable.

High HSP90 expression was repeatedly demonstrated in breast cancer patients, but this only applies to ductal carcinoma, as in the case of the lobular subtype, a clear decrease in HSP90 expression was found [40]. The study of the heterogeneity of breast cancer showed a correlation of increased expression of several genes responsible for HSP90 synthesis with an increased risk of death and aggressive cancer phenotype. Different HSP90 isoforms are associated with negative prognosis for various neoplastic disease subtypes. It was suggested that increased levels of HSP90 may be a prognostic factor for aggressive neoplasms (HER2/ER2 status) with higher risk of recurrence and distant metastases [41].

Noteworthy is the correlation of the extracellular level of HSP90 with the EMT of neoplastic cells, which is a sign of neoplastic progression [42]. This is confirmed by morphological changes in cancer cells corresponding to the mesenchymal phenotype – for example, elongated shape, loss of cell-cell junctions and the acquisition of the ability of cells to migrate, and thus transformation from tight cubic epithelial cells pattern into loosely dispersed groups. This is accompanied by changes in the expression of EMT markers (E-cadherin, N-cadherin, Zeb1, Zeb2). The decisive role of ex-HSP90-LRP1 (lipoprotein receptor-related protein – LRP1) in the initiation of the migration capacity of prostate cancer cells has been confirmed. Increased levels of ex-HSP90 are associated with the aggressive neoplastic cell phenotype, which prompts research into the possibility of using ex-HSP90 in cancer diagnosis and treatment [43]. The prognostic value of HSP90 in gastric and colorectal cancer has also been confirmed, its high expression indicating positive correlation with invasiveness and metastasis [44]. HSP90 was suggested to be an indicator of KN differentiation in lung adenocarcinoma [45]. Of the 4 isoforms of HSP90 that are present in humans, 2 of them – GRP94 and TRAP1 – show higher expression compared to the others – HSP90α and HSP90β – in patients with small cell lung cancer. The diversified expression of these isoforms indicates their different participation in carcinogenesis [46]. Increased expression of Hsp90β can be used for the differential diagnosis of pleural effusion in lung cancer and correlates with pathomorphological differentiation, tumour size, and lymph node metastases as well as the aggressive course of the disease [47].

The presented results indicate the purposefulness of research into the role of HSPs in the early diagnosis of cancer and the importance of HSPs as biological markers of cancer progression.

Heat shock proteins as a target in anti-cancer therapy

Considering the importance of HSPs at various stages of the neoplastic process, they seem to be an interesting therapeutic target. The development of HSP inhibitors is, however, quite a challenge due to the lack of selectivity towards neoplastic and normal cells, and therefore high toxicity, as well as the problem of removing them from the cell by means of membrane transporters responsible for the phenomenon of multidrug resistance (MDR).

Geldanamycin (GA), an antibiotic from the ansamycin family, was the first HSP90 inhibitor. Studies have shown that GA stops tumour proliferation by inhibiting the activity of Src tyrosine kinase and blocking the ATP binding site in HSP90, leading to proteasomal degradation of these proteins [48]. The structure of GA was then modified and 17-AAG (tanespimycin) was developed. 17-AAG was the first HSP90 inhibitor to be used in human clinical trials. In addition, in vivo studies were carried out to test 17-AAG as a tool to induce apoptosis and inhibit cell proliferation by increasing the concentration of cytochrome c, caspase-9, and caspase-3 [49]. The significant anti-neoplastic effect allowed 17-AAG to enter phase I and II clinical trials in patients with advanced breast cancer, pancreatic cancer, and melanoma, where it was used both as monotherapy and in combination with gemcitabine, cisplatin, bortezomib, and trastuzumab [50–52].

Other GA derivatives such as 17-DMAG, IPI-504 (re-tasipimycin) and IPI-493 were also tested [53]. Following the success of phase I and II clinical trials, IPI504, as well as other HSP90 inhibitors such as STA-9090 and AUY922, were subjected to phase III studies in the non-small cell lung cancer (NSCLC) population. Their efficacy has not been demonstrated in a random population of NSCLC patients [54–56].

However, the results of studies on another HSP90 inhibitor, dorsomorphin, are encouraging. It was demonstrated that dorsomorphin reduces HSF1 Ser320 phosphorylation and nuclear translocation, as well as the level of nuclear HSF1 in cancer cells, inducing their apoptosis [57]. Moreover, in the conducted in vitro studies, it sensitized cancer cells to HSP90 and proteasome inhibitors and inhibited HSP70 expression induced by these inhibitors. In mice, dorsomorphin enhances neoplastic cell apoptosis and inhibits HSP70 expression and tumour growth.

The phenomenon of multi-drug resistance is a serious limitation of many anti-cancer therapies and is the subject of ongoing research. Eleven HSP90 inhibitors containing an isoazolonaphthoquinone core have been synthesized and tested in tumour models of small cell lung cancer and MDR colorectal adenocarcinoma [58]. The efficacy was determined on the basis of the inhibition of cell growth, ac-
tivity, and expression of P-glycoprotein (P-gp) — the membrane transporter responsible for MDR. For some of the tested compounds, interaction with P-gp was observed through inhibition of its ATPase activity and decreased P-gp expression in cancer cells.

In order to obtain selectivity of HSP inhibitors towards neoplastic cells, attempts were made to combine HSP90 inhibitors with anti-cancer drugs or radiotherapy, as well as to deliver compounds to the cancer cells, e.g., in nanosomes (drug delivery systems) [7]. The anti-neoplastic activity of the combination of the HSP90 XL888 inhibitor and vemurafenib was demonstrated in patients with melanoma with the BRAFV600 mutation [59].

It was demonstrated that the new derivative of 5-re-sorcinol triazolone (PTP-Ganetesib) was active in breast cancer cells, including MDA-MB-231 triple-negative breast cancer, and it was approved for phase III clinical trials [60].

5-aryl-3-thiophene-2-yl-1H-pyrazole, another new HSP90 inhibitor, was tested on MCF7 and HepG2 (hepatocellular carcinoma) cell lines. The compound containing the thio-phene group showed the highest antiapoptotrophic and apoptotic activity against HepG2 cells. It caused cell cycle arrest in the G2 phase, a 7.7-fold increase in caspase-3, in-apoptotic activity against HepG2 cells. It caused cell cycle arrest in the G2 phase, a 7.7-fold increase in caspase-3, and decreased Hsp90 levels by 70.8%. Moreover, it significantly decreased the level of Hsp90 client proteins (Akt, c-Met, c-Raf, and EGFR) and gave a 1.57-fold increase of Hsp70 [61].

VER155008, a new inhibitor that targets HSP70, blocks the phosphorylation of the PI3K/AKT/mTOR and MEK/ERK signalling pathways. VER155008 may reduce the proliferation of PC12 pheochromocytoma and induce apoptosis, as well as inhibit cell migration and invasion [62]. In addition, VER-155008 inhibits the proliferation of breast and colon cancer lines (HCT116 and BT474) and induces the degradation of HSP90. Induction of caspase-3/7-dependent apoptosis in BT474 cells and caspase-independent death of HCT116 cells was also observed.

2-Phenylethylsulfonamide (PES) is a selective inhibitor of HSP70 function in various types of KN, showing lower toxicity to normal cells. PES was shown to inhibit the proliferation of oral squamous cell carcinoma cell lines in vivo and in vitro, arrest the cell cycle arrest, and trigger apoptosis. PES inhibited the expression of X-linked inhibitor of apoptosis protein (XIAP), c-IAP1, p-AKT, and p-ERK and disrupted the interaction between Hsp70 and XIAP [63].

Recently, it was found that JG-98, an inhibitor containing pyridinic acid modified benzothiazole, showed promising antiproliferative activity in cancer cells. However, pyridinic acid causes undesirable disturbances in biochemical and cellular reactions. Therefore, it has been replaced with neutral pyridine. Pyridine-modified benzothiazoles, such as JG2-38, retain promising antiproliferative effects in breast and prostate cancer cell lines [64].

Cantharidin (CTD), a terpenoid derivative, inhibits HSP70 expression by blocking HSFl binding to the HSP70 promoter [65]. The introduction of thermostensitive CTD closing liposomes induces cell apoptosis, blocking the response to heat shock and subsequent expression of HSP70 and BAG3 in cervical cancer [66].

Apoptozole (AZ) is an inhibitor of HSP70 that promotes neoplastic cell apoptosis by permeabilising the lysosomal membrane. AZ-mediated disruption of lysosomal function also inhibits protective autophagy and promotes cell apoptosis in multiple cancer cell lines [67].

The assessment of the effect of dihydroartemisinin (DHA) on HSP70 expression in PC-3 prostate cancer cells has demonstrated that DHA inhibits HSP70 expression in PC-3 cells. DHA interferes with the formation of the association between dATP cytochrome c, Apaf-1, caspase-3, apoptosis-inducing factor, and HSP70, and consequently inhibits the anti-apoptotic capacity of HSP70 and promotes apoptosis of PC-3 cells [68].

The search for inhibitors targeting HSP27 is extremely difficult because HSP27, unlike other heat shock proteins, is independent of ATP [69]. However, a new strategy for inhibiting HSP27 by inducing cross-linking of the HSP27 protein has been suggested. Introducing inserts into the disulphide bond alters the cross-linking of HSP27, which interferes with HSP27 dimerization and function. Moreover, altered HSP27 dimerization may sensitize cancer cells that express HSP27 [70].

Of the HSP27 inhibitors, OGX-427, an antisense oligonucleotide (apartorssen), is the most prominent. Its effectiveness has been assessed in preclinical and clinical trials [71]. High therapeutic activity of OGX-427 was demonstrated in combination with traditional anti-cancer drugs (gemcitabine, docetaxel) [72,73], as well as with other chaperone inhibitors and proteotoxic agents, demonstrating significant efficacy. OGX-427, co-administered with the autophagy inhibitor chloroquine, significantly inhibited the growth of prostate cancer in animal models [74]. Complex administration of the Hsp90 inhibitor PF-04928473 together with OGX-427 revealed effective inhibition of cancer cell growth and induction of apoptosis. In a castration-resistant prostate cancer (CRPC) model, the above combination inhibited tumour growth and prolonged animal survival [75].

Bortezomib significantly inhibited HSP27 expression. Treatment with bortezomib or OGX-427 inhibited proliferation and promoted apoptosis in U266 cell lineage and significantly reduced HSP27 expression. Moreover, Bcl-2 expression was significantly decreased, while BAX expression was increased by bortezomib and OGX-427. There was no significant difference between the bortezomib group and OGX-427 in vitro. HSP27 correlates positively with Bcl-2 expression and negatively with BAX expression in U266 cells. Bortezomib promotes apoptosis in multiple myeloma cells [76]. Recently, research on ivermectin as an inhibitor of HSP27 capable of enhancing oncogene targeting in cancer models has been presented [77].

Conclusions
Numerous experimental studies confirm the assumption of using HSPs as clinical biomarkers and therapeutic targets in oncology. HSPs have been shown to be overexpressed in some types of cancer and to play an important role in oncogenic processes, and, therefore, they may be of great prognostic value. HSPs are associated with process-
es typical of cancer development and progression, such as cell proliferation, invasion, and metastasis. In addition, HSPs influence cell survival mechanisms by interacting with key apoptotic proteins. This allows them to be regarded as promising targets for various therapies. The inhibition of HSP with various preparations is the subject of research in a number of cancers. However, more research is needed to refine the efficacy of HSP in combination with other traditional markers for cancer diagnosis and prognosis of their progression.

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