Heat Shock Protein 70 Inhibitors

Subjects: Oncology

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A class of chaperones dubbed heat shock protein 70 (Hsp70) possesses high relevance in cancer diseases due to its cooperative activity with the well-established anticancer target Hsp90. However, Hsp70 is closely connected with a smaller heat shock protein, Hsp40, forming a formidable Hsp70-Hsp40 axis in various cancers, which serves as a suitable target for anticancer drug design.

Hsp70 heat shock proteins cellular Hsp40 anticancer agents

1. Introduction

Stress factors such as heat lead to defensive and protective cellular responses, enabling the cell to cope with the stress [1]. The emergence of chromosomal puffs as a defined heat shock response in *Drosophila* flies was observed for the first time in 1962 by Ferruccio Ritossa [2]. In 1974, the increased expression of certain heat shock proteins in response to heat and other stress factors was discovered [3]. These heat shock proteins (Hsps) are classified by their molecular weights in kDa (e.g., Hsp90, heat shock protein 70 (Hsp70), etc.) and function as chaperones in order to protect important proteins from degradation, to control the quality of protein folding, and to deliver misfolded or damaged proteins to the proteasome for disposal (protein triage), thus vouchsafing cell viability under these conditions [4]. This multi-chaperone system (“epichaperome”) plays an important role in various cancer diseases [5]. Canonical functions of chaperones are linked with the ubiquitin-proteasome system (UPS) and chaperone-mediated autophagy (CMA), while non-canonical functions affect the immune system, including inflammatory and autoimmune mechanisms [6]. Thus, targeting heat shock proteins is a promising strategy to combat cancer.

The heat shock protein (Hsp) family is subdivided into Hsp110 (HSPH), Hsp90 (HSPC), Hsp70 (HSPA), Hsp40 (DNAJ), small Hsps (HSPB), and the chaperonin family proteins Hsp60/Hsp10 (HSPD/E) and TRiC (CCT) [7]. Hsp90 has already become a valuable chaperone drug target. Prominent examples of anticancer active Hsp90 inhibitors are the natural products (geldanamycin and radicicol) as well as their (semi-)synthetic derivatives, 17-AAG/tanespimycin and ganetespib, the latter have reached clinical trials [8]. Hsp90 regulates the activity and stability of crucial transcription factors such as the tumor suppressor p53 and the androgen receptor (AR), while Hsp90 activity itself is regulated by posttranslational modifications (e.g., lysine acetylation under control by HDAC6) and by other heat shock proteins such as Hsp70 [9]. The protein folding by Hsp90 and Hsp70 is ATP-dependent, while Hsp70 has a crucial function in the protection of cells against various stress factors, including enhanced cell survival. In addition, Hsp70 forms complexes with Hsp90 with the help of HOP (Hsp70–Hsp90 organizing protein) in order to exert its housekeeping activities [10][11][12]. There is growing evidence that Hsp70 inhibitors have the

potential to overcome Hsp90 inhibitor resistance [13]. The organelle-specific members of the Hsp70 protein family, such as mitochondrial mortalin (mtHsp70, Grp75) and endoplasmic reticulum/ER-based Grp78, were also identified as possible anticancer drug targets due to their crucial roles in cell proliferation and survival in various cancers [14][15]. In addition, extracellular Hsps attracted increased attention as cancer targets and biomarkers [16].

Client polypeptides are transferred to Hsp70 by the smaller co-chaperone, Hsp40. Hsp40 prevents the aggregation of unfolded polypeptides, but there are also folded proteins among the clients of Hsp40. There are numerous Hsp40 isoforms, also dubbed J-domain proteins (DNAJs), that are structurally different from Hsp70 and Hsp90 proteins [17]. Nevertheless, DNAJ/Hsp40 and Hsp70 proteins form a tight Hsp70/Hsp40 complex in order to fulfill their protein-folding functions [17][18]. Hsp40 binds to the nucleotide-binding domain (NBD) of Hsp70 and accelerates the ATPase activity of Hsp70 enormously [18]. Thus, small-molecule inhibitors of the Hsp70-Hsp40 axis have considerable potential as anticancer drug candidates.

2. Inducible Heat Shock Protein 70 and Heat Shock Cognate 71 Kilodalton Protein Inhibitors

Inducible Hsp70 (also abbreviated as Hsp70i) and consecutive Hsc70 inhibitors can be classified according to their binding mode into N-terminal nucleotide binding domain/NBD-targeting inhibitors, C-terminal substrate binding domain/SBD-targeting inhibitors, and allosteric inhibitors (**Table 1**).

In 2009, the adenosine-derivative **1** (**Figure 1**, VER-155008) was identified as a selective NBD-targeting Hsc70/Hsp70 inhibitor (IC_{50} = 0.5 µM), which showed antiproliferative effects on HCT-116 colon carcinoma cells (GI_{50} = 5.0 µM), reduced Raf-1 and Her2 protein levels, and enhanced the apoptosis induction by the Hsp90 inhibitors 17-AAG and VER-82160 in HCT-116 cells [19][20]. Compound **1** also revealed promising effects on non-small cell lung cancer (NSCLC), such as inhibition of NSCLC proliferation and cell cycle arrest (increased G0/G1 cell percentage) [21]. In addition, compound **1** inhibited pleural mesothelioma cell proliferation and colony formation, which are associated with G1 cell cycle arrest, suppressed phospho-Akt, and induction of macroautophagy [22]. In LNCaP95 prostate cancer cells, the Hsp70 inhibitor **1** induced apoptosis and suppressed the expression of the full-length androgen receptor (AR-FL) and of the androgen receptor splice variant 7 (AR-V7), which are associated with castration-resistant prostate cancer (CRPC) [23]. These AR-suppressing effects were correlated with an inhibition of YB-1 phosphorylation by compound **1**, followed by reduced nuclear translocation of YB-1. Another study using the AR-positive LNCaP and the AR-negative PC-3 prostate cancer cell lines showed that compound **1** was antiproliferative and pro-apoptotic in both cell lines, albeit the pro-apoptotic effects were higher in the AR-positive cells [24]. Compound **1** downregulated AR expression and induced G1 cell cycle arrest in the LNCaP cells. Moreover, a distinct suppression was observed for Hsp27 in PC-3 cells and HOP and Hsp90β in both cell lines treated with compound **1**. In MCF-7 breast cancer cells, compound **1** induced apoptosis associated with mitochondrial damage, and the anticancer activity of compound **1** was reduced by heat shock [25]. Anaplastic thyroid carcinoma (APC) is the most lethal thyroid cancer with high drug resistance, but compound **1** was able to induce paraptosis in APC cells dependent on de novo protein synthesis [26]. In a panel of glioma cells (1321N1, GOS-3, and U87-MG), compound **1** showed higher antiproliferative activities (IC_{50} = 12–13 µM) than the approved

drug temozolomide (IC_{50} = 135–180 μM) associated with downregulation of Akt kinase activity and the modulation of certain miRNAs, e.g., the upregulation of miR-215 and miR-194-5p [27]. Compound **1** was evaluated in muscle-invasive bladder cancer (MIBC) models and induced apoptosis along with inhibition of MIBC cell proliferation and migration [28]. The activity of compound **1** against MIBC was associated with the suppression of protein members of p53/Rb, PI3K, and SWI/SFW signaling. Especially strong degrading effects of compound **1** were observed on the demethylase KDMA6 and the histone acetyltransferase EP300, both members of the histone modification pathway.

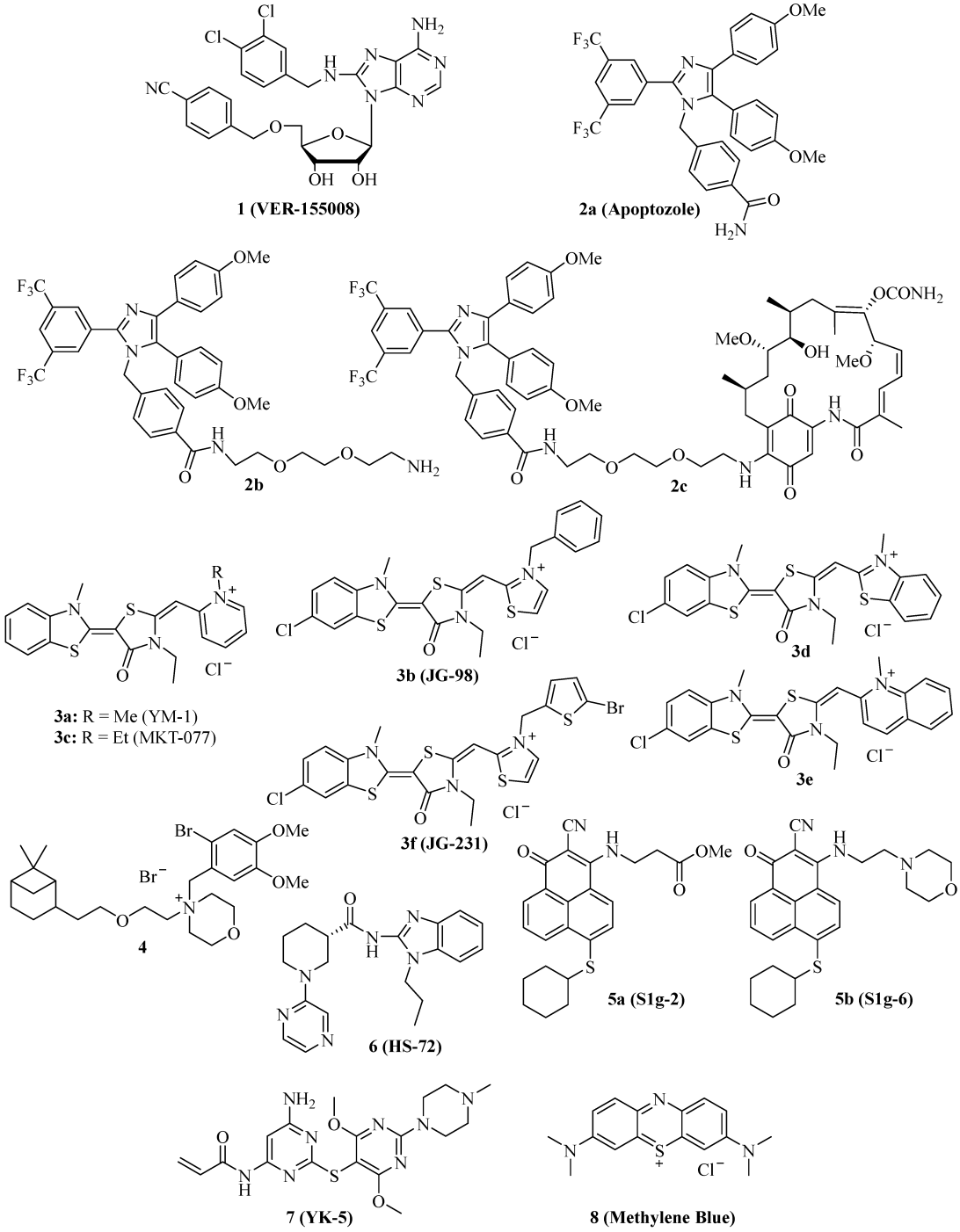

Figure 1. Structures of NBD-binding and interfering compounds **1**–**8**.

Due to these promising anticancer effects of compound **1**, its combination with various other anticancer drugs was investigated. In combination with the Hsp90 inhibitor radicicol, compound **1** was able to enhance the APC cell killing activity of radicicol, accompanied by suppressed heat shock cognate 70/Hsc70, Akt, and survival [29]. The combination of compound **1** with the Hsp90 inhibitor STA9090 was evaluated in MIBC models and was more efficient than single compound therapy [28]. However, the combination of compound **1** with doxorubicin in canine osteosarcoma (OSA) cells showed no improvements. Already, compound **1** alone displayed strong apoptosis induction, inhibition of colony formation, and antiproliferative activities against OSA cells based on Hsp70 inhibition as well as Akt suppression and BAG1 degradation [30]. In contrast to that, compound **1** showed synergy effects in combination with the Hsp90 inhibitor 17-AAD and sensitized A549 NSCLC cells to radiation therapy [21]. The combination of compound **1** with manumycin A, an anticancer active antibiotic that upregulates Hsp70 in cancer cells, sensitized lung tumor cells to manumycin A treatment [31]. Micelles of compound **1** together with gold nanorods were successfully tested as mild-temperature photothermal therapy in colon cancer, leading to strong colon tumor growth in vivo at a temperature of 45 °C [32]. Compound **1** exhibited considerable activity against multiple myeloma (MM) cells (IC_{50} = 1.7 µM for OPM2, 3.0 µM for RPMI 8226, and 6.5 µM for MM.1S cells), and the combination of compound **1** with the proteasome inhibitor bortezomib displayed synergy effects in terms of apoptosis induction in MM cells, which was associated with suppression of anti-apoptotic Bcl-2, Bcl-xL, and Mcl-1 and upregulation of pro-apoptotic NOXA and Bim [33]. In addition, the ER stress marker CHOP (CCAAT-enhancer binding protein homologous protein) was induced by this combination treatment. The natural product shikonin was described as a proteasome inhibitor and necroptosis inducer in MM cells while it also upregulated Hsp70, and, thus, the combination with compound **1** enhanced shikonin-induced MM cell death [34]. Compound **1** also exhibited promising effects on acute myeloid leukemia (AML) cells alone (induction of apoptosis, inhibition of cell proliferation, and colony formation) and in combination with the Hsp90 inhibitor 17-DMAG (additive antiproliferative and pro-apoptotic activity) [35]. The release of AML cell growth factors and regulators such as TNF-α, VEGF, IL-3, IL-1β, and IL-1 receptor antagonist was strongly suppressed upon treatment with compound **1**. A new structurally related 6,8,9-trisubstituted purine derivative was recently disclosed that induced apoptosis and senescence in luminal A subtype MCF-7 breast carcinoma cells [36].

The substituted imidazole derivative **2a** (**Figure 1**, apoptozole) was discovered as a pro-apoptotic inhibitor of Hsc70 (K_D = 210 nM) and Hsp72 (K_D = 140 nM) in 2008, which showed high tumor cell growth inhibitory activities with GI_{50} values in the nanomolar concentration range (GI_{50} = 220 nM for SK-OV-3, 250 nM for HCT-15, and 130 nM for A549 cells) [37]. Compound **2a** was identified as an inhibitor of the Hsp70 ATPase by affinity chromatography upon conjugation of the amino-ethyloxy modified apoptozole derivative **2b** (**Figure 1**) with a resin, leading to a reduced interaction of Hsp70 with APAF-1, while no affinity to Hsp40, Hsp60, or Hsp90 was observed [38]. Time-dependent antiproliferative activity was checked, and amenable IC_{50} values (0.8 µM for A549, 0.8 µM for HeLa, 0.7 µM for MDA-MB-231, and 0.7 µM for HepG2 cells) were obtained after 72 h of incubation, while the combination with doxorubicin led to a sensitization of A549 and HeLa cells to doxorubicin treatment. At doses of 4 mg/kg/day (i.p.) given for two weeks, compound **2b** reduced the tumor growth of A549, RKO, and HeLa tumor xenografts by 61%, 65%, and 68%, respectively; in the latter case, the combination with doxorubicin led to more pronounced tumor growth inhibition (81% tumor growth reduction). The anti-leukemia properties of compound **2b** and its hybrid

molecules, such as compound **2c** (**Figure 1**), with the Hsp90 inhibitor geldanamycin (**2b** and geldanamycin connected by ethylene glycol-based linker systems) were studied [39]. Compounds **2b**, hybrid **2c,** and other related hybrids induced apoptosis in a caspase-dependent way, however, the hybrids were more active against leukemia cells than their parent compounds **2b** and geldanamycin. Compound **2c** inhibited autophagy, but it also induced apoptosis in HeLa cancer cells based on its selective inhibition of the lysosomal Hsp70 and the degradation of lysosomal membranes associated with cathepsin release followed by caspase activation [40].

Several synthetic allosteric inhibitors of the Hsp70 ATPase domain were described. The rhodacyanine class of the Hsp70-inhibitory dye compounds was established by the discovery of compound **3a** (**Figure 1**, YM-1), in particular, by the blocking of the Hsp70 interaction with the nucleotide exchange factor (NEF) Bag3 by compound **3a** (IC_{50} = 4.8 µM) [41]. **3a** binds to an allosteric binding site, stabilizing ADP-bound Hsp70 with a weak Bag3 affinity. Consequently, compound **3a** suppressed FoxM1 and HIF1α pathways in MCF-7 and HeLa cells, which is unique for Hsp70-Bag3 inhibition since other Hsp70 inhibitors (e.g., the natural flavonoid myricetin) did not show such mechanistic effects. In MCF-7 breast carcinoma xenografts, compound **3a** (25 mg/kg every second day for 6 days, i.p.) inhibited tumor growth associated with induction of p21 and suppression of FoxM1 and survivin. The activity of compound **3a** was also investigated in glioma models, and it sensitized U251 and U343 glioma cells to treatment with the Bcl-2 inhibitors (-)-gossypol (AT-101) and ABT-737 [42]. Analogously, **3a** sensitized apoptosis-resistant and chemo-resistant breast cancer cells to drug treatment [43]. In doxorubicin-resistant BT-549^{r}DOX cells, compound **3a** suppressed Mcl-1 and showed synergistic antiproliferative effects in combination with doxorubicin. In addition to compound **3a**, further close Hsp70-inhibitory analogs such as compound **3b** (**Figure 1**, JG-98) with thiazolium moieties were described, which were discovered during the search for optimized Hsp70 inhibitors derived from compound **3a** and the mortalin (mitochondrial Hsp70) inhibitor MKT-077 (**Figure 1**, compound **3c**, see below). Compound **3b** exhibited prolonged microsomal half-lives and at least 3-fold higher antiproliferative activities against MDA-MB-231 triple-negative breast cancer (TNBC; EC_{50} = 0.4 µM) and hormone-sensitive MCF-7 breast cancer cells (EC_{50} = 0.7 µM) than compounds **3a** (EC_{50} = 2.0 for MDA-MB-231 and 5.2 for MCF-7 cells) and **3c** (EC_{50} = 1.4 for MDA-MB-231 and 2.2 for MCF-7 cells) [44]. Compound **3b** induced apoptosis in MDA-MB-231 cells by caspase activation and enhanced p62 oligomerization as a hint at forming autophagosomes. Compound **3b** also inhibited the Hsp70-Bag3 interaction, which was accompanied by FoxM1 suppression and upregulation of p21 and p27 in treated MCF-7 cells [45]. Doses of 3 mg/kg (every second day for six days) of compound **3b** inhibited the growth of MCF-7 breast carcinoma xenografts in vivo. In addition to breast cancer cell lines MCF-7 and MDA-MB-231, further cancer cell lines sensitive to compound **3b** were identified (with EC_{50} < 1 µM), such as HeLa, A375, HT-29, SKOV-3, Jurkat, MM1.R, INA6, RPMI-8226, JJN-3, and U266. Mechanistically, compound **3b** exerted its antiproliferative activities against breast cancers both in Bag3-dependent (via ERK activation) and Bag3-independent ways (via suppressed Akt and *c*-Myc) [46]. Combinations with the proteasome inhibitors MG132 and bortezomib enhanced the antitumor activity of compound **3b** in breast cancer models both in vitro and in vivo (5 mg/kg of compound **3b** plus 1 mg/kg of bortezomib in MDA-MB-231 TNBC xenografts). Synergy effects in breast cancer cells were also observed in combination with α-amanitin (RNA-polymerase II inhibitor), LY294002 (Akt inhibitor), and sunitinib (RTK inhibitor). In addition, the infiltration of tumor bodies by tumor-associated macrophages (TAMs) was inhibited by compound **3b** based on the inactivation of tumor stromal cell Hsp70

proteins [47]. Similar to compound **1**, compound **3b** also sensitized lung cancer cells to treatment with manumycin A [31]. In a recent effort, new rhodacyanine analogs of compound **3b** with benzo-fused *N*-heterocycle moieties were described, with benzothiazolium compound **3d** and quinolinium compound **3e** (**Figure 1**) as the most promising compounds exhibiting high antiproliferative activity against TNBC cells (IC_{50} = 0.24 µM for **3d** and 0.37 µM for **3e** in MDA-MB-231 cells), accompanied by a considerable selectivity since non-malignant MCF-10A cells with low levels of Hsp70 were much less sensitive to treatment with **3d** [48]. Both compounds are very stable, with half-lives of more than 2 h in microsomes. Apoptosis upon caspase-activation was induced by compounds **3d** and **3e** in MDA-MB-468 breast cancer cells, while both compounds led to autophagy both in MDA-MB-231 and MDA-MB-468 cells. In addition to FoxM1, survivin, HuR, and Akt suppression, compounds **3d** and **3e** also degraded KRAS in MDA-MB-231 and MDA-MB-468 cells. Further **3b**/JG-98-derived benzothiazole rhodacyanines were described, culminating in the discovery of the bromothienyl analog **3f** (JG-231), which showed high activity against MCF-7 (IC_{50} = 0.12 µM) and MDA-MB-231 breast cancer cells (IC_{50} = 0.25 µM), disruption of Bag3 interaction, amenable microsomal stability (half-life of more than 60 min), degradation of Akt and HuR in MCF-7 xenografts, amenable in vivo pharmacokinetics parameters and in vivo tumor growth inhibition of MDA-MB-231 xenografts at doses of 4 mg/kg (i.p.) [49].

The cationic spasmolytic drug pinaverium bromide (**Figure 1**, **4**) was repurposed as an inhibitor of constitutively activated Hsc70. Compound **4** inhibited cell proliferation (IC_{50} of ca. 10 µM) of A2058 melanoma cells and induced apoptosis in these cells [50]. Its binding site was located at the NBD and linker domains of Hsc70.

S1g-2 (**Figure 1**, **5a**) was identified as an inhibitor of Hsp70-Bim interaction (IC_{50} = 0.4 µM) in CML cells by screening a Bcl-2 inhibitor library [51]. Its allosteric Hsp70-binding site is near the binding site of the rhodacyanines **3**. Compound **5a** selectively induces apoptosis in CML cells by suppressing oncoprotein clients such as Akt, Raf-1, eIF4E, and RPS16. Hsp70-Bim interaction protected BCR-ABL-independent TKI-resistant CML cells from apoptosis, and, thus, treatment with compound **5a** can overcome the resistance of a highly problematic CML type. The close analog compound **5b** (**Figure 1**, S1g-6), which has a morpholino side chain replacing the unstable ester side chain of **5a**, was recently described as a new sub-micromolar Hsp70-Bim interaction inhibitor [52]. Compound **5b** also induced apoptosis in cancer cells and suppressed Akt and Raf-1.

The benzimidazole derivative **6** (**Figure 1**, HS-72) selectively inhibits inducible Hsp70 (Hsp70i), which is in stark contrast to its low affinity for the closely related constitutively activated Hsc70 [53]. At doses of 25 and 50 µM, compound **6** inhibited the proliferation of BT474, MCF-7, and SkBr3 breast cancer cells, and the proteins Her2 and Akt were degraded by compound **6** in BT474 and MCF-7 breast cancer cells. In the Her2-overexpressing MMTV-*neu* spontaneous breast tumor mouse model, compound **6** (20 mg/kg biweekly for 21 days, i.p.) was well tolerated and inhibited breast tumor growth, leading to prolonged survival of treated mice.

The 2,5′-thiodipyrimidine **7** (**Figure 1**, YK-5) was identified as an irreversible inhibitor of a new allosteric site of the Hsp70 NBD domain and binds covalently to a cysteine residue of the binding site via its reactive acrylamide moiety [54]. It is highly active against Kasumi-1 AML cells (IC_{50} = 0.9 µM) and SkBr3 breast cancer cells (IC_{50} = 0.8 µM)

and a potent apoptosis inducer by caspase-3/7 activation (IC_{50} = 1.2 µM) in MOLM13 AML cells. **7** degraded Her2 and Raf-1 in SkBr3 breast cancer cells as a consequence of Hsp70 inhibition.

The dye methylene blue (**Figure 1**, **8**) showed manifold biological activities, and, thus, it was also identified as an inhibitor of the Hsp70 ATPase, leading to a rapid suppression of Tau protein in neurodegenerative cell models [55]. In HeLa cervix carcinoma cells expressing poly-glutaminylated AR (AR112Q), compound **8** inhibited the Hsp70-mediated degradation of AR112Q [56]. In A375 and G361 metastatic melanoma cells, compound **8** suppressed the heat shock response (downregulation of Hsp27, Hsp70, and Hsc70), induced ROS formation, and caused glutathione depletion at a concentration of 10 µM [57]. Geldanamycin is an Hsp90 inhibitor, which increases Hsp70 expression, but compound **8** (10 µM) was able to suppress geldanamycin-induced Hsp70 expression in A375 melanoma cells. Hence, 10 µM of compound **8** also sensitized A375 melanoma cells to geldanamycin treatment, but it also sensitized these cells to treatment with etoposide and doxorubicin. Compound **8** possessed antiproliferative properties against A549 NSCLC cells and induced early apoptosis, yet it enhanced the degradation of N-terminal AR fragments as well as autophagy [58]. In mice, compound **8** inhibited benzo[a]pyrene induced lung carcinogenesis and suppressed Hsp70 as well as the tumor biomarkers ADA and LDH.

The structures of competitive and allosteric NBD binders are shown in **Figure 1**.

Table 1. Effects of NBF-targeting Hsp70 inhibitors on cancers.

Compound	Cancer Model(s)	Effects
1 (VER-155008)	HCT-116 colon carcinoma, A549 NSCLC, pleural mesothelioma, LNCaP95 prostate carcinoma, anaplastic thyroid carcinoma, glioma (1321N1, GOS-3, U87-MG), muscle-invasive bladder cancer, osteosarcoma, multiple myeloma, acute myeloid leukemia	Selective Hsp70/Hsc70 inhibition, antiproliferative, suppression of Her2 and Raf-1, G1 cell cycle arrest, sensitization to 17-AAG and radiation, suppression of Akt and phospho-Akt, macroautophagy induction, suppression of AR-FL and ARV7, apoptosis and paraptosis induction, upregulation of miR-215 and miR-194-5p, degradation of KDMA6 and EP300, degradation of BAG1, upregulation of CHOP, suppression of VEGF release by leukemia cells, synergy effects with drugs (manumycin A, bortezomib, shikonin, 17-DMAG) and PDT [19][20][21][22][23][24][25][26][27][28][29][30][31][32][33][34][35]
2a (Apoptozole)	SK-OV-3 ovarian carcinoma, HCT-15 colon carcinoma, A549 NSCLC	Suppression of Hsp70-APAF-1, antiproliferative, pro-apoptotic [37]
2b, **2c**	HeLa cervix carcinoma, MDA-MB-231 breast carcinoma, HepG2 hepatoma, A549 NSCLC, RKO colon carcinoma, leukemia	Suppression of Hsp70-APAF-1, antiproliferative, sensitization to doxorubicin, in vivo tumor growth inhibition of A549, RKO, and HeLa xenografts, apoptosis induction, autophagy inhibition, cathepsin release [38][39][40]
3a (YM-1)	MCF-7 breast carcinoma, HeLa cervix carcinoma, U251 and U343 glioma, doxorubicin-	Inhibition of Hsp70-Bag3, suppression of FoxM1 and HIF1α pathways, in vivo inhibition of MCF-7 tumor growth, induction of p21, suppression of FoxM1 and surviving,

Compound	Cancer Model(s)	Effects
	resistant BT-549^{r}DOX breast carcinoma	sensitization of glioma to (−)-gossypol (AT-101) and ABT-737, suppression of Mcl-1, synergistic antiproliferative effects with doxorubicin [41][42][43]
3b (JG-98)	Triple-negative MDA-MB-231 and hormone sensitive MCF-7 breast cancer, lung cancer, miscellaneous	Inhibition of Hsp70-Bag3, FoxM1 suppression, upregulation of p21 and p27, sensitization of breast cancer to bortezomib in vivo, inhibition of TAM infiltration, sensitization of lung cancer to manumycin A [31][44][45][46][47]
3d, **3e**	Triple-negative breast cancer (e.g., MDA-MB-231, MDA-MB-468)	Stable, antiproliferative, tumor-selective, induction of apoptosis and autophagy, degradation of KRAS, suppression of FoxM1, survivin, HuR, and Akt [48]
3f (JG-231)	MCF-7 and MDA-MB-231 breast cancer	Stable, antiproliferative, inhibition of Hsp70-Bag3, degradation of Akt and HuR, tumor growth inhibition in vivo [49]
4 (Pinaverium bromide)	A2058 melanoma	Apoptosis induction [50]
5a (S1g-2)	CML	Inhibition of Hsp70-Bim, apoptosis induction, suppression of Akt, Raf-1, eIF4E and RPS16 [51]
5b (S1g-6)	Miscellaneous	Inhibition of Hsp70-Bim, apoptosis induction, degradation of Akt and Raf-1 [52]
6 (HS-72)	BT474, MCF-7 and SkBr3 breast carcinoma, Her2-overexpressing MMTV-*neu* spontaneous breast tumor mouse model	Selective Hsp70i inhibition, antiproliferative, Her2 and Akt degradation, tumor growth inhibition and prolonged survival in vivo [53]
7 (YK-5)	Kasumi-1 AML, SkBr3 breast carcinoma, MOLM13 AML	Antiproliferative, apoptosis induction, Her2 and Raf-1 degradation [54]
8 (Methylene Blue)	AR112Q-expressing HeLa cervix carcinoma, A375 and G361 melanoma, A549 NSCLC	Heat shock response suppression, ROS formation, glutathione depletion, suppressed geldanamycin-induced Hsp70, sensitization of cancer cells to geldanamycin, etoposide and doxorubicin, apoptosis induction, inhibition of lung carcinogenesis in vivo [56][57][58]

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ts Hsp70-

related effects in various cancers. Compound **9a** showed antiproliferative activities against various osteosarcoma, breast, and pancreatic carcinoma cell lines at IC_{50} values of 5–10 µM independent of the p53-state of the tumor cells, induced cell death independent of caspase activation, and led to dysfunctional autophagy by the formation of p62-oligomers/aggregates [59]. It decreased the interaction of Hsp70 with APAF1, p53, and the co-chaperones Hsp40, CHIP, and BAG-1M and suppressed NF-κB signaling and activity. In vivo, compound **9a** (40 mg/kg, i.p., every five days for 30 days) blocked Myc-based lymphopathogenesis and led to prolonged survival in Eµ-Myc transgenic mice. Compound **9a** exhibited considerable antiproliferative activity against acute leukemia (AML and ALL) cells (IC_{50} = 2.5–12.7 µM), induced apoptosis in these cells by caspase activation, and led to a degradation of Akt and ERK1/2 [60]. In addition, compound **9a** sensitized acute leukemia cells to treatment with cytarabine (an antimetabolite), 17-AAG (a Hsp90 inhibitor), vorinostat (a HDAC inhibitor), and sorafenib (a RTK inhibitor). In

primary effusion lymphoma (PEL), compound **9a** exerted cytotoxic effects on BC3 and BCBL1 cells by apoptosis and another cell death mechanism, which was associated with immunogenic activity such as activation of dendritic cells [61]. Compound **9a** increased lysosome permeabilization and cathepsin D release in PEL cells, accompanied by Bid cleavage, outer mitochondrial depolarization, and AIF translocation to the nucleus. Moreover, compound **9a** sensitized PEL cells to bortezomib treatment. The combination of compound **9a** with DNA-targeting platinum complexes such as cisplatin and oxaliplatin also revealed synergy effects in HT-29 colon carcinoma and PC-3 prostate carcinoma cells [62]. In prostate cancer cells, compound **9a** also increased the antitumor effects of hyperthermia (HT, 43 °C), best when given immediately before HT started, which was accompanied by upregulation of p21 and suppression of c-Myc and cyclin D1 [63]. The combination of compound **9a** (100 µg in 50 µL) and HT (43 °C for 1 h, twice on days 0 and 4), led to significant PC-3 prostate carcinoma xenograft growth inhibition. Its modified analog compound **9b** (**Figure 2**, PES-Cl) was antiproliferative against a panel of BRAF-V600E mutant melanoma (IC_{50} values between 2–5 µM, while inactive against melanocytes) and showed higher antiproliferative activity than compound **9a** against SkBr3 breast carcinoma, FaDu head and neck squamous cell carcinoma, and H1299 lung adenocarcinoma cells [64]. The cytotoxic activity of compound **9b** is based on apoptosis induction (caspase activation) and inhibition of autophagy (p62 accumulation), while the HeLa cell cycle was arrested in the G2-M phase by compounds **9a** and **9b** associated with cyclin B1 degradation. At doses of 20 mg/kg (i.p., once per week), compound **9b** led to a much higher survival rate of Eµ-Myc mice (71.4% survival) than compound **9a** (35% survival) after 210 days. Compound **9b** also induced apoptosis in A375 melanoma cells, accompanied by Her2 degradation in these cells [65]. In contrast to compounds **1** and **3c**, only compound **9b** led to G2-M arrest in H1299 and A375 cells based on cyclin B1 degradation.

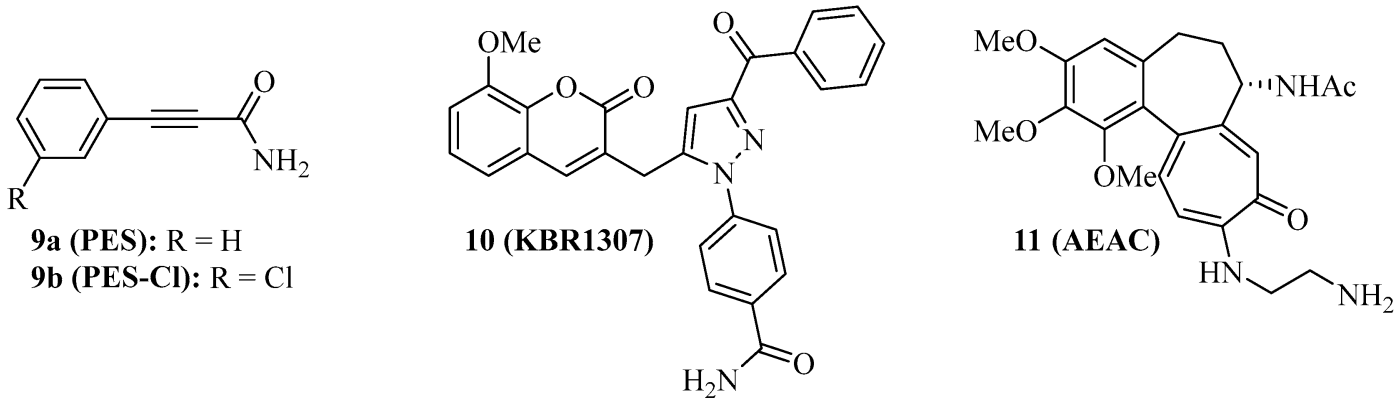

Figure 2. Structures of SBD-interacting Hsp70 inhibitors **9**–**11**.

Compound **9a** does not interact with Hsp70 when no nucleotide is bound to the protein. The coumarin-pyrazole hybrid, compound **10** (**Figure 2**, KBR1307) was designed, which binds Hsp70 in the presence and absence of nucleotides [66]. Both compounds **9a** and **10** reduce the activity of Hsp70 ATPase significantly, but compound **10** was more active against MCF-7 cells than compound **9a**. Similar coumarin-thiazole hybrids were described by the same group before as binders to the C-terminus of Hsp70 with activity against DLD-1 colon carcinoma and HepG2 hepatoma cells [67].

The screening of the InterBioScreen compound library for Hsp70 inhibitors revealed that the semi-synthetic colchicine derivative, compound **11** (**Figure 2**, *N*-aminoethylaminocolchicine, AEAC), interferes with substrate binding and refolding functions of Hsp70, based on a nanomolar affinity for Hsp70 (K_D = 149 nM) [68]. Although the antiproliferative and cytotoxic activities of compound **11** are low, it sensitized C6 rat glioblastoma and B16 mouse melanoma cells to doxorubicin treatment. The combination of compound **11** (2 mg/kg) and doxorubicin (1 mg/kg) inhibited in vivo B16 tumor growth by 71% and increased the lifespan of treated mice by ca. 15 days when compared with untreated mice.

The structures of SBD-interacting compounds are shown in **Figure 2**. **Table 2** summarizes the anticancer effects of the described SBD-domain interacting Hsp70 inhibitors.

Table 2. SBD-interacting Hsp70 inhibitors and their effects on cancer model(s).

Compound	Cancer Model(s)	Effects
9a (PES)	Colon, breast, prostate and pancreas carcinoma, osteosarcoma, lymphoma, acute leukemia	Antiprolierative independent from p53-state, caspase activation, dysfunctional autophagy, prolonged survival of Eμ-Myc mice, NF-κB suppression, degradation of Akt and ERK1/2, immunogenic activity, sensitization of cancers to drugs and hyperthermia [59][60][61][62][63]
9b (PES-Cl)	BRAF-V600E mutant melanoma, SkBr3 breast carcinoma, FaDu head and neck squamous cell carcinoma, H1299 lung adenocarcinoma, lymphoma	More antiproliferative than **9a**, apoptosis induction, autophagy inhibition, G2-M phase arrest, degradation of cyclin B1 and Her2, prolonged survival of Eμ-Myc mice [64][65]
10 (KBR1307)	MCF-7 breast carcinoma	More antiproliferative than **9a**, binds Hsp70 in absence of nucleotide [66]
11 (AEAC)	C6 rat glioblastoma, B16 mouse melanoma	Increased doxorubicin activity in vitro and in vivo, tumor growth inhibition (71%) and prolonged survival in B16 mice [68]

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