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Review

Combination therapies with HSP90 inhibitors against colorectal cancer

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ABSTRACT

Oncogene stability and homeostasis mediated by the HSP90 chaperone is a crucial protection trait of cancer cells. Therefore, HSP90 represents an attractive therapeutic target for many cancers, including colorectal cancer. Although monotherapy has limited clinical efficacy, preclinical and early-phase clinical studies indicate improved antitumor activity when HSP90 inhibitors are combined with chemotherapies or targeted agents. This may be further improved with a biomarker-guided approach based on oncogenic HSP90 clients, or stratification based on the consensus molecular subtypes of colorectal cancer, suggesting a synergistic activity with 5-fluorouracil in preclinical models of the chemorefractory mesenchymal subtype. Furthermore, HSP90 inhibition may activate mechanisms to turn non-immunogenic tumors hot and improve their recognition by the immune system, suggesting synergy with immune checkpoint blockade.

1. A "chaperone-view"

The term chaperone may loosely be used as a common denominator for both the function of the heat shock protein 90 (HSP90) and its current interest as a target for anti-cancer agents. As will be discussed, the clinical promise is likely to be realized by combination therapies, and it is particularly exciting that HSP90 inhibition provides an alternative way to treat cancers with targets that are "difficult to drug" directly, or it may even bypass therapy resistance of well-established drugs [1–3]. The HSP90 protein itself is a chaperone in the conventional interpretation of the term and plays a central role in preventing misfolding of several hundreds of downstream "clients" [4].

Colorectal cancer (CRC) is a major cancer type that affects both genders, and approximately half of the patients will die within 5 years of their diagnosis. Patients with metastatic disease have a 5-year overall survival at around only 10% [5], and although some benefit from standard oncological treatment and surgery, there is great potential to improve precision oncology for this heterogeneous disease. New treatment strategies with molecularly targeted therapies and immune checkpoint inhibition show promise to improve survival for small patient subgroups [5,6]. However, a transition from a one-gene, one-drug approach to a multi-molecular, multi-therapeutic model is called for [7,8]. This rationale strengthens the potential of multi-drug regimens including agents that operate by different mechanisms to reduce the possibility of resistance development.

In this review, we provide a brief overview of the multifactorial downstream effects of HSP90 activity and outline promising clinical applications of combination therapies with HSP90 inhibitors in CRC, with the aid of appropriate biomarkers for patient stratification.

2. Multifaceted downstream targets of HSP90

Living cells depend on constant maintenance of cellular proteostasis to withstand the continuous exposure to various stress factors [9,10]. Correct protein folding mediated by molecular chaperones is one of the basic stress response mechanisms [10], and in response to temperature stress in particular, the heat shock proteins (HSPs) are triggered for this purpose. A central player in this cascade is HSP90, which structure and function has recently been reviewed (see Ref. [11]).

Due to dependency on continued oncogenic signals, cancer cells are especially susceptible to proteotoxic stresses, making HSPs highly involved in their pathophysiology [10]. Many oncoproteins that contribute to accelerated growth, proliferation and drive the survival of neoplastic cells are clients of HSP90, and consequently cancer cells may be more susceptible to inhibition of HSP90 than normal cells [4,12,13]. Indeed, HSP90 is highlighted as a potential therapeutic target in the

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treatment of cancers that are driven by oncoproteins such as HER2, BRAF, EML4-ALK, EGFR, CDK4, CRAF, AKT, MET, and BCR-ABL [13], making HSP90 an attractive target for drug development and motivating an armamentarium of inhibitors that are currently developed (see Ref. [13,14]).

HSP90 has also gained attention as an important factor in the extracellular space, denoted "eHSP90", where it is involved in the regulation of tumor invasiveness and metastasis [15]. It is not clear whether HSP90 targeting with small molecules primarily affects intracellular or extracellular functions of HSP90, but there are attempts to target eHSP90 specifically with novel cell impermeable drugs in early development. Extracellular HSP90 is also detected on the surface of secreted exosomes, which are cell-derived vesicles that play various roles in tumor growth, metastasis, drug resistance and immune response [16]. A recent study has even described that Hsp90 in Drosophila (78% similarity with human HSP90) controls exosomal release by supporting the fusion of multivesicular bodies with the plasma membrane [17]. Accordingly, it is conceivable that HSP90 inhibition may influence the heterotypic interactions of the tumor cells with the tumor-associated stroma.

2.1. Chaperoning oncogenic pathways in CRC

HSP90 clients regulate cancer-critical mechanisms such as angiogenesis, metastasis, apoptosis, and drug resistance [18]. Indeed, several oncogenic pathways, including transforming growth factor β (TGF-β), mitogen-activated protein kinases (MAPKs), AKT/PI3K, and WNT, are influenced by HSP90 mediated stabilization [19] (Fig. 1). Of particular clinical relevance to CRC, receptor tyrosine kinases (RTKs) and several of their signaling transducers depend on HSP90 chaperonage. Blockade of the epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF) and its corresponding RTK receptor VEGFR, have clinical benefit and are part of standard treatment for patients with metastatic CRC [5,8]. As will be discussed later, preclinical and clinical studies have shown that HSP90 inhibitors are promising combination partners to RTK blockade, suggesting a clinical relevance for HSP90 inhibition in CRC [18].

KRAS GTPase is a prominent mediator of mitogenic signaling of many RTKs (Fig. 1). Around 40% of CRCs carry activating mutations in the *KRAS* oncogene, and they are (together with *NRAS* mutations) used in the clinic as negative predictive biomarkers for anti-EGFR therapy [5,20]. Furthermore, *KRAS* mutations also have negative prognostic associations, which may be subtype-dependent in primary CRC [21]. Noteworthy, KRAS itself is not an HSP90 client, but preclinical studies of cell lines from CRC and other cancer types have shown that mutated *KRAS* might predict sensitivity to HSP90 inhibition due to serine/threonine kinase 33 (STK33) depend mechanisms [2,3]. Since HSP90 is a chaperone for STK33, HSP90 inhibitors have been proposed to have therapeutic efficacy in *KRAS* mutated cancers [22]. However, in the only completed phase II clinical trial with HSP90 inhibition in CRC, which investigated single agent treatment with ganetespib in 17 metastatic cancers refractory to chemotherapy, stable disease was the best treatment response, in only two out of nine patients with *KRAS* mutated cancers [2] (Table 1). No activity was observed in the remaining patients, and no post-treatment changes in the expression of HSP90 client proteins were demonstrated. The authors proposed that too infrequent dosing of ganetespib to effectively degrade client proteins might explain the lack of activity [23].

KRAS activates the downstream protein kinase BRAF, which in itself is a prominent oncoprotein in cancer and a well-known HSP90 client [1,14] (Fig. 1). *BRAF* (V600E) activating mutations are mutually exclusive of *KRAS* mutations and are found in a poor prognostic subgroup of approximately 10% of CRCs [24]. *BRAF* mutations are more common in microsatellite unstable than microsatellite stable CRCs, but in the

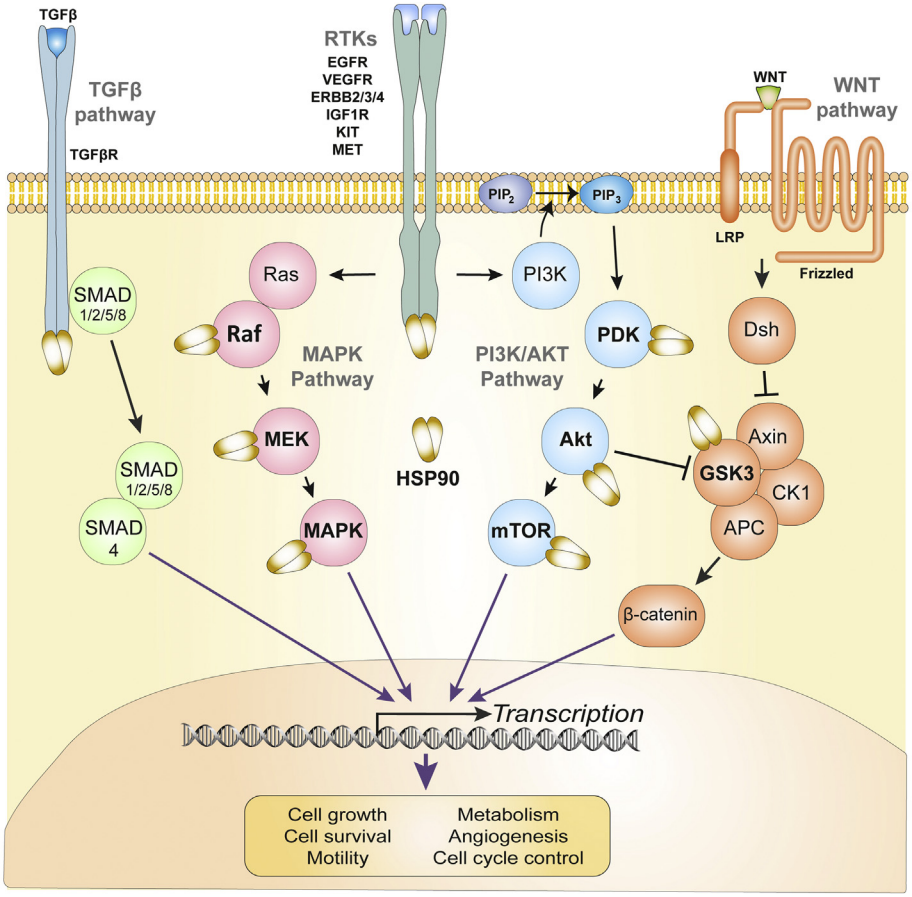

Fig. 1. HSP90 chaperonage of oncogenic pathways in CRC. Four major oncogenic pathways that drive growth, proliferation and invasiveness of CRC are simplified and depicted in distinct colors. Receptor and downstream proteins marked in bold followed by an HSP90 molecule indicate signaling mediators that are known HSP90 clients in CRC. HSP90 – Heat shock protein 90; TGF-β – Transforming growth factor beta; TGFβR – TGF-β receptor; SMAD – Mothers against decapentaplegic homolog; EGFR – epidermal growth factor receptor; VEGFR – vascular endothelial growth factor receptor; ERBB – erythroblastic oncogene B; IGF1R – Insulin-like growth factor 1 receptor; KIT – KIT Proto-oncogene receptor tyrosine kinase; MET – MET proto-oncogene receptor tyrosine kinase; Ras – Kirsten rat sarcoma viral proto-oncogene; Raf – Raf proto-oncogene serine/threonine protein kinase; MEK – Mitogen-activated protein kinase kinase 1; MAPK – Mitogen-activated protein kinase; PI3K – Phosphoinositide 3-kinase; PIP2 – Phosphatidylinositol 4,5-bisphosphate; PIP3 – Phosphatidylinositol (3,4,5)-trisphosphate; PDK – Pyruvate dehydrogenase kinase; Akt – Protein kinase B; mTOR – Mechanistic target of rapamycin; Wnt – Proto-oncogene Wnt; LRP – Lipoprotein receptor-related protein; Dsh – Dishevelled protein; GSK-3 – Glycogen synthase kinase 3; CK1 – Casein kinase 1; APC – Adenomatosis polyposis coli.

Table 1
Clinical studies including CRC patients testing HSP90 targeting agents.

Drug formulation	Phase/ recruitment status	Condition	Outcome measure/response	Secondary outcome measures	Clinicaltrials.gov trial annotation and study
Ganetespib	Phase I Completed	Advanced solid malignancies incl. CRC	42/53 evaluable patients 23 – stable disease One patient with mCRC had a partial response	HSP70	NCT00687934 Goldman, J.W. et al. (2013)
	Phase I Completed	Refractory mCRC	17 evaluable patients No overall response 2 – stable disease (*KRAS* mut)	pERK, pAKT, CyclinD1, HIF-1α, VEGFR2, p70S6, HSP70. *KRAS, BRAF, PIKC3A*	NCT01111838 Cercek, A. et al. (2014)
Ganetespib + Capecitabine and Radiation	Phase I Completed	Stage II/III rectal cancer	15/16 evaluable patients 3/12 – pathologic complete response 2 – residual tumor 6/9 – clearing of lymph node disease	Half-life of ganetespib and capecitabine	NCT01554969 El-Rayes, B.F. et al. (2015)
Ganetespib + Ziv-aflibercept	Phase I Terminated	Progressive malignancies incl. mCRC	4/5 evaluable patients 3 stable disease 1 progressive disease	HIF-1α, EGFR	NCT02192541 Meehan, R.S. et al. (2018)
Luminespib + Capecitabine	Phase I Completed	Advanced solid malignancies incl. CRC	19/23 evaluable patients 4 partial response 8 stable disease 7 progressive disease	Drug related toxicities	NCT01226732 Bendell, J.C. et al. (2015)
Luminespib + Cetuximab	Phase IB Completed	KRAS wt mCRC	15/16 evaluable patients 1 – partial response 4 – stable disease	Half-life of luminespib	NCT01294826 Subramaniam, S. et al. (2015)
Tanespimycin + Irinotecan	Phase I Completed	Advanced solid malignancies incl. CRC	22/27 evaluable patients no complete or partial responses 11 – stable disease	HSP70, pH2AX, pChk1, p-histone H3, cl. caspase 3, p53	NCT00119236 Tse, A.N. et al. (2008)
Tanespimycin + Sorafenib	Phase I Completed	Advanced solid malignancies incl. CRC	23/28 evaluable patients 2 – partial response 14 – stable disease stable disease in 1/4 CRC patients	Half-life of tanespimycin and sorafenib. HSP90, HSP70, CDK4, pAKT, pERK, RAF1, β-actin	NCT00121264 Vaishampayan U.N. et al. (2010)
PEN-866 (HSP90 targeting ligand linked to SN-38)	Phase I/IIA Recruiting	Advanced solid malignancies incl. CRC	Phase I – determine the dose and toxicity. Phase II – determine ORR	Half-life of PEN-866 and its metabolites. Radiographic PFS. OS.	NCT03221400
XL888 + Pembrolizumab	Phase IB Recruiting	Advanced GI malignancies incl. CRC	Determine the dose and toxicity of the combination	Immune profile in the serum and in tumor biopsies	NCT03095781

Clinicaltrials.gov accessed on 20 October 2018
CRC – colorectal cancer, mCRC – metastatic colorectal cancer, PFS – progression free survival, OS – overall survival; ORR – objective response rate, GI – gastrointestinal.

primary setting, the negative prognostic associations may be limited to the latter group [21,25]. The negative predictive role of *BRAF* mutations in wild-type *KRAS* towards anti-EGFR treatment remains inconclusive [26]. Due to feedback mechanisms, monotherapy with BRAF inhibition is not effective in CRC, but dual inhibition of EGFR and BRAF, or triple inhibition of EGFR, BRAF, and MEK have shown efficacy in patients [24,27]. HSP90 inhibitors in combination with BRAF inhibitors display superior activity compared to monotherapy in targeting mutant *BRAF* melanoma in preclinical- and clinical studies [1,28]. In CRC, the clinical efficacy of HSP90 inhibitors has not been studied in relation to *BRAF* mutation status, but preclinical findings nominate a subset of BRAF-driven CRCs sensitive to chaperone inhibition [1,29]. However, another preclinical study conducted in monolayer and three-dimensional CRC cell line cultures showed that *BRAF* mutation was a resistance factor against luminespib, proposing co-inhibition of both HSP90 and BRAF for better efficacy [30].

3. Limited efficacy of HSP90 inhibitor monotherapy

Consistent with the lack of a meaningful antitumor activity of ganetespib in the phase II study of metastatic CRC (Table 1), clinical efficacy of HSP90 inhibition as a single agent has been limited. Currently, no HSP90 inhibitors have been approved for clinical use, despite broad clinical efforts to prove their efficacy in several cancer types [4,14]. Many clinical trials have been terminated or postponed due to only moderate effects of the studied inhibitors (clinicaltrials.gov). The best clinical efficacy with HSP90 inhibitor monotherapy has been achieved in tumors strongly addicted to particular client oncoproteins, such as ALK in a subtype of non-small cell lung cancers and in HER2-overexpressing breast cancers [14,31].

General resistance mechanisms to HSP90 inhibitors have been described and may provide clues to improve their efficacy. Heat shock factor protein 1 (HSF1) activity is broadly recognized as a key resistance factor of HSP90 inhibition [32,33]. HSF1 binds HSP90 and is released to form active homotrimers after HSP90 inhibition (with N-domain inhibitors), thereby inducing a prosurvival heat shock response [33,34]. Additionally, overexpression of the multidrug resistance efflux pump P-glycoprotein 1 (P-gp) bypasses the anti-cancer activity of benzoquinone-based HSP90 inhibitors [34], whereas high expression of UDP glucuronosyltransferase 1A (UGT1A) is associated with resistance to resorcinol-based inhibitors such as ganetespib and luminespib [23,29]. These proteins might restrain the activity of HSP90 inhibitor monotherapy, but switching to C-terminal HSP90 inhibitors or tailored co-inhibition strategies have been shown to reverse the resistance [29,34,35].

A recent study found that chaperone conglomeration (termed epichaperome) is predictive of sensitivity to HSP90 inhibition, rather than the expression levels of individual members of the epichaperome (HSP90, HSC70, HOP, HSP110, CDC37, AHA1), HSP90 clients, anti-apoptotic proteins and genetic alterations [36]. Interestingly, this

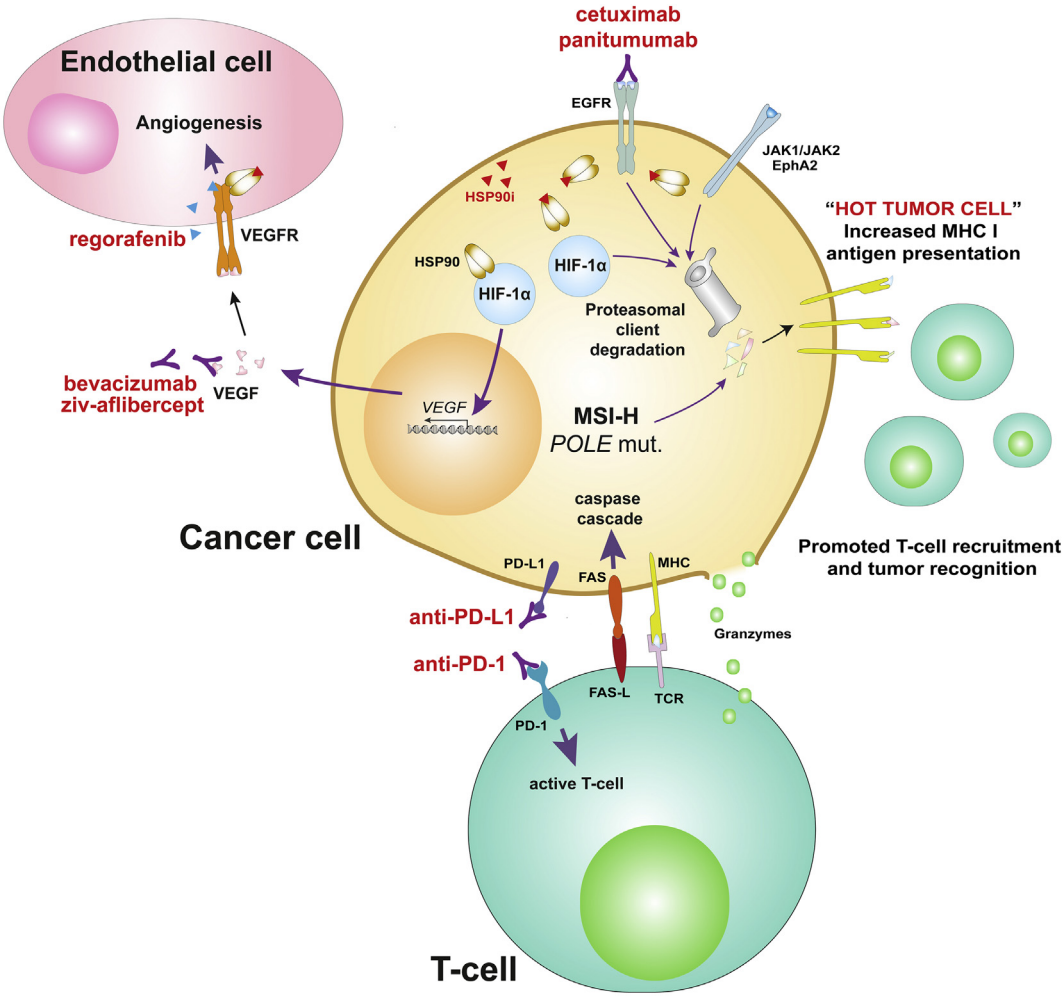

Fig. 2. HSP90 inhibition can improve targeted therapy and immunotherapy in CRC. Targeted treatment of EGFR-driven CRC is managed by applying anti-EGFR antibodies cetuximab and panitumumab which induce receptor internalization and degradation. Neoangiogenesis VEGF/VEGFR pathway in CRC tumors is blocked using anti-VEGF antibodies bevacizumab or ziv-aflibercept that sequester VEGF, prohibiting it from binding to the receptor as well as inhibiting VEGF/VEGFR signaling with the tyrosine kinase inhibitor regorafenib. Checkpoint inhibitor antibodies anti-PD1 and anti-PD-L1 allow activation of T-cell-mediated anti-cancer effects via FAS receptor-mediated apoptosis and granzyme mediated cancer cell lysis. Increased MHC I mediated antigen presentation improves T-cell recruitment that is fostered by intracellular protein degradation and high-mutational load from MSI phenotype or mutated POLE, thus turning cancer cells immunogenic or "hot". HSP90 inhibitors depicted as red triangles hinder the stabilization process of various HSP90 clients which in turn end in the MHC-I neo-antigen presentation pathway. HSP90 – Heat shock protein 90; EGFR – Epidermal growth factor receptor; HIF-1α – Hypoxia-inducible factor 1-alpha; VEGF – Vascular endothelial growth factor; VEGFR – Vascular endothelial growth factor receptor; MSI-H – Microsatellite instability-high; POLE – Polymerase epsilon; PD-1 – Programmed cell death protein 1, PD-L1 – Programmed death ligand 1; MHC-I – Major histocompatibility complex class 1; TCR – T-cell receptor; FAS – First apoptosis signal receptor; FAS-L – FAS ligand; JAK1/2 – Janus kinase 1/2; EphA2 – Ephrin type-A receptor 2.

finding suggests that patients can be stratified based on the abundance of the epichaperome and not solely its existence. This favorable epichaperome for HSP90 inhibition therapy was detected in 60–70% of cell lines derived from breast-, lung-, pancreatic- and gastric cancers, as well as leukemia and lymphomas [36]. Nevertheless, the epichaperome formation has not been investigated as a potential predictive factor for HSP90 inhibition in CRC.

4. HSP90 inhibitors in combination therapies

Lessons learned from clinical investigations and emerging studies in preclinical models suggest that HSP90 inhibitors can potentiate the effect of other anti-neoplastic treatments, including targeted agents, conventional chemotherapies, radiotherapy, and immunotherapy [1,29,37]. Clients of HSP90 that mediate drug resistance against currently approved drugs in CRC are of particular interest for co-therapeutic approaches, and combination treatments that are investigated in clinical studies involve both conventional chemotherapies and targeted agents (Table 1).

4.1. HSP90 inhibitors in combination with chemotherapy

Pre-clinical data suggest that HSP90 inhibition can potentiate the effect of all chemotherapies used in standard-of-care for CRC, including 5-fluorouracil (5-FU) [32], irinotecan [38,39] and oxaliplatin [40], but combinations have been clinically tested only with the former two. Despite new entries in the anticancer armamentarium, the fluoropyrimidine 5-FU remains a mainstay therapy for patients with CRC, both in the adjuvant and metastatic setting [5]. 5-FU is an antimetabolite that acts by disrupting DNA and RNA synthesis and repair *via* incorporation into the nucleotide sequence, as well as by inhibition of thymidylate synthase [41]. The latter, if overexpressed or amplified, acts as a resistance factor against 5-FU [41]. HSP90 inhibition leads to down-regulation of thymidylate synthase and thus synergizes with fluoropyrimidine-based chemotherapy, as shown in CRC cell lines and xenograft models [29,32,42]. A phase I clinical study testing

luminespib and capecitabine (peroral 5-FU pro-drug) in 19 patients, mostly with advanced CRC, resulted in partial response or stable disease for 63% of the enrolled subjects (Table 1).

Rectal cancers are commonly treated with chemoradiation prior to surgery, to facilitate complete surgical resection and reduce the risk of local recurrences. Similar to several chemotherapeutics, ionizing radiation introduces excessive DNA damage that results in cell death. Several preclinical studies have provided compelling evidence for radiosensitization activity of HSP90 inhibitors in several cancer types, including CRC [43]. Potential modes of action include inhibition of cell cycle checkpoint activation and DNA double-strand break repair, which subsequently lead to induction of cell cycle arrest, apoptosis and necrosis, as well as decreased cell migration and invasiveness [43–45]. Clinical potential for the combined use of radiation therapy and HSP90 inhibition has been shown in a phase I study, where capecitabine and radiation therapy was combined with ganetespib as neoadjuvant treatment in stage II and III rectal cancers, achieving pathologic complete response in 25% of the patients, in addition to lymph node clearance in the resected specimen in 67% (Table 1).

SN-38 is the active metabolite of irinotecan that inhibits topoisomerase I, the unwinding enzyme required during DNA replication, resulting in irreversible DNA damage and cell death [44]. Irinotecan is part of the combinatorial conventional chemotherapy regimens FOLFIRI or FOLFOXIRI, commonly used as first-line treatment for patients with metastatic CRC [5]. Inhibition of topoisomerase I by SN-38 leads to G2/M cell cycle arrest that is regulated by several proteins such as the DNA response mediators Serine/threonine-protein kinase Chk1 (CHEK1) and Wee1-like protein kinase (WEE1) [39]. Both CHEK1 and WEE1 are HSP90 clients, and their depletion with the HSP90 inhibitor tanespimycin proved to increase the cytotoxicity of SN-38 in TP53-defective cells [39]. This observed effect was then investigated in a phase I clinical study in 27 patients with different solid tumor types treated with irinotecan and tanespimycin [38]. Six of the enrolled subjects were CRC patients, and stable disease was the best response observed. In 16 patients with known *TP53* status, stable disease was measured predominantly in patients with *TP53* mutated (five out of ten) compared to wild-type tumors (two out of six). Although there was no subsequent phase II trial on this combination, possibly due to the introduction of newer HSP90 inhibitors, another clinical study was initiated with the novel PEN-886 conjugate in patients with advanced solid malignancies (Table 1). PEN-886 (STA-8666) is a miniature conjugate of an HSP90 targeting moiety linked to SN-38 [46] that has shown promising preclinical activity in other cancer models [47,48].

4.2. HSP90 inhibitors in combination with targeted therapy

Among the few targeted therapies used in standard-of-care for metastatic CRC are the monoclonal anti-EGFR antibodies cetuximab and panitumumab, as well as bevacizumab, regorafenib, and Ziv-aflibercept that block angiogenesis mediated by VEGF/VEGFR signaling in the endothelium [49] (Fig. 2).

HSP90 inhibitors are investigated as potential candidates for combination therapies with both EGFR- and VEGF-targeting agents and have been tested in preclinical- and clinical studies for various cancer types, including CRC [50–54].

The EGFR protein and its downstream mediators, several of which are negative predictors of anti-EGFR therapy, require HSP90 chaperonage; hence, HSP90 presents a promising therapeutic approach to overcome anti-EGFR treatment resistance. Combination of luminespib with cetuximab was assessed in an early phase clinical study of patients with *KRAS* wild-type metastatic CRC [50]. One patient showed a partial response (6%), and four other patients had stable disease (25%); thus, this treatment regimen should be further explored in CRC (Table 1).

Furthermore, a phase I clinical study investigated the activity of tanespimycin in combination with the multi-kinase inhibitor sorafenib that targets VEGFR, PDGFR, and kinases of the RAF family [55]. The rationale for this combination was to enhance kinase inhibition and protein degradation (due to HSP90 inhibition) of RAF isoforms that might appear as a consequence of insufficient targeting with sorafenib single treatment. Although there were only four CRC patients treated with this combination in the study, one of these patients had a stable disease. Partial response ($n = 2$) and stable disease ($n = 14$) was achieved in nine renal cancer patients (75%) and four melanoma patients (67%), suggesting further development of this combination in the respective diseases [55]. Part of these observed responses was suggested to be due to prevention of angiogenesis, recognizing that both drugs target this pathway.

The role of HSP90 in the regulation of angiogenesis is well established. In addition to chaperoning the pro-angiogenic VEGFR2, PRKD2, STK33, and STAT3 [56–58], HSP90 was shown to regulate the hypoxia-induced factor (HIF-1α) that in turn promotes angiogenesis *via* VEGF expression (Fig. 2) [56,58,59]. Following these preclinical studies, a phase I study investigated the combination of ganetespib and Ziv-aflibercept in patients with progressive malignancies [54]. Among the five enrolled patients, three had stable disease, one progressed and another was not evaluable (Table 1). The maximum tolerated dose as the primary outcome measured was not reached due to unfavorable toxicity profile and early trial closure. Accordingly, the clinical benefit for this combination strategy is inconclusive.

4.3. HSP90 inhibition may improve the effect of immunotherapy

Elucidation of anticancer mechanisms of immune checkpoint inhibitors in recent years has led to the development of T-cell-based immunotherapies such as anti-cytotoxic T lymphocyte antigen-4 (CTLA-4) and anti-programmed cell death protein 1 (anti-PD-1) antibodies that proved effective in many malignancies [60]. Most CRCs do not benefit from immunotherapy, but a subset of hypermutated metastatic CRCs with microsatellite instability (MSI; 3–4%) show a good overall response [61] (Fig. 2). These hypermutated tumors are characterized by defects in DNA mismatch repair (MMR) and have a high neo-antigen burden, making them highly immunogenic, commonly referred to as “hot tumors” [62,63] (Fig. 2). In addition to the MSIs, another small subset of hypermutated CRCs, caused by mutations in the DNA polymerase epsilon (*POLE*) or delta (*POLD1*) genes, are attractive for immunotherapy [61] (Fig. 2). However, approximately half of the hypermutated cancers do not respond to immunotherapy, and only a few resistance mechanisms have been identified [60].

In melanoma cells, a list of 850 bioactive drugs was incubated prior to co-culture with autologous tumor-infiltrating T cells (TILs) in a high-throughput screen which revealed HSP90 inhibitors as top candidates to increase the sensitivity of tumor cells to T-cell killing [37]. Furthermore, HSP90 inhibition was shown to potentiate antineoplastic activities of anti-CTLA4 or anti-PD-1 therapy *in vivo*. The synergism in T-cell-mediated anti-tumor immune response after HSP90 inhibition was based on the upregulation of the IFN-induced protein with tetratricopeptide repeat (IFIT) gene family [37]. Increased expression of these interferon response genes is associated with long-term benefits to anti-CTLA4 immunotherapy across multiple cancer types [64]. In addition, several HSP90 clients have been shown to regulate immune checkpoint blockade *via* induction of PD1 and PD-L1 expression, including mutated EGFR, rearranged ALK, HIF-1α, and JAK2 [65]. In another preclinical study, syngeneic mouse models bearing colon carcinoma (MC38) and melanoma (B16) tumors treated with a combination of ganetespib and STI-A1015 (anti-PD-L1 antibody) displayed a greater antitumor activity as compared to single drugs or vehicle [65]. In the CRC model, the HSP90 inhibitor SNX-5422 in combination with monoclonal antibodies against PD-1, PD-L1 or CTLA4 demonstrated an improved antitumor activity supporting further clinical development of this combination regimen against CRC [66].

Another rationale for a synergistic effect between HSP90 inhibition and immunotherapy is increased antigen presentation of oncogenic

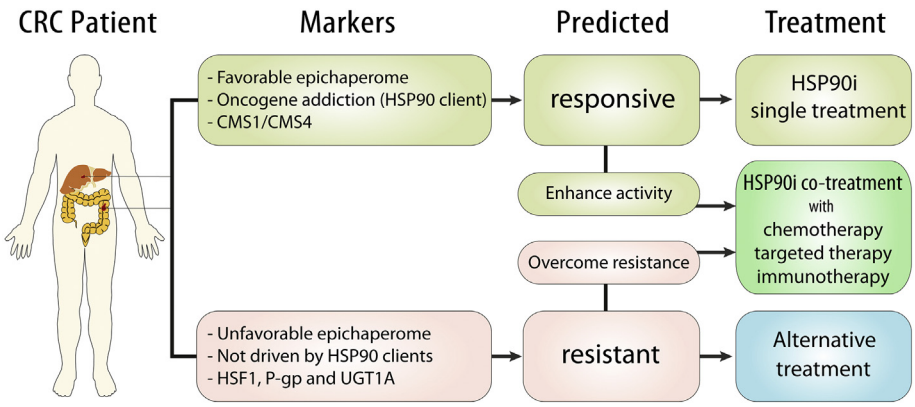

Fig. 3. Stratification of CRC for HSP90 inhibition. Indicated are traits that predict sensitivity (light green) and resistance (red) to HSP90 inhibitors based on preclinical findings. HSP90 inhibitors are encouraged to combine with other drugs (dark green) to enhance their anticancer activity or bypass resistance, although, the clinical effectiveness remains to be further studied. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

clients upon proteasome degradation after HSP90 inhibition. This may turn non-immunogenic tumors "hot" by attracting or activating infiltrating immune cells against the tumor cells (Fig. 2). For example, the EphA2 tyrosine kinase receptor, which is commonly expressed in a variety of cancer types, is a well-known client of HSP90 that promotes malignant growth and invasiveness [67]. Inhibition of HSP90 induces the degradation of tumor EphA2, followed by an enhanced MHC-dependent presentation of EphA2 antigens to EphA2-specific CD8+ T cells [67]. These EphA2-specific CD8+ T cells have been detected in the peripheral blood of patients with prostate cancer, glioma and renal cell carcinoma [68,69]. Combination of HSP90 inhibitors with agonists of EphA2 was shown to further enhance the antigen presentation and tumor cell recognition by the anti-EphA2 CD8+ T cells [70].

Improved effects of immunotherapy might also come from targeting the tumor stroma. Tauriello et al. recently examined the role of the TGF-β-activated tumor microenvironment in immunosuppression of CRC metastases [71]. They conclude that inhibition of TGF-β signaling could be implemented in immunologically cold tumors as a pro-immunogenic therapy. Although the tumor suppressor role of TGF-β in epithelial cancer cells discourages inhibition of TGF-β as cancer treatment [72], in CRC metastases with intact TGF-β, inhibiting this pathway might eradicate the disease by unleashing the immune system [71]. Hypothetically, turning cold tumors with active TGF-β signaling hot might be achieved with HSP90 inhibition as well, as both TGF-β receptors TGFβR1 and 2 are HSP90 clients [73] (Fig. 1).

5. Patient stratification to improve the effect of HSP90 inhibition in combination therapies

Although combination therapies including HSP90 inhibitors represent an attractive therapeutic strategy, clinical efficacy is likely to be improved with accompanying biomarkers to identify tumors that are sensitive to chaperone-targeted therapy [11] (Fig. 3).

Considering the limited clinical testing of HSP90 inhibition in CRC, comprehensive analyses of potential predictive factors are pending. However, pre-clinical studies may provide important clues, as has been discussed above for prominent oncogenes such as *KRAS* and *BRAF*.

Moving towards the multi-molecular approach in precision medicine, consensus molecular subtypes (CMS) provide a new biological framework that classify CRCs independently of cancer stage [7,8]. CMS is a transcriptomic classification system that reflects distinct biological traits of primary CRC tumors and appears to have prognostic value in early-stage and advanced disease, as well as a predictive value based on the retrospective analysis of clinical trials [7,8]. The epithelial-like canonical CMS2 subtype is particularly sensitive to EGFR and HER2 blockade, owing to their frequent overexpression of EGFR and amplification of *ERBB2* [7,8,32]. In contrast, the mesenchymal-like CMS4 subtype is associated with a lack of response to oxaliplatin and EGFR-inhibitors [74,75]. Recently, in a preclinical study using 33 CRC cell lines, we performed a high-throughput drug screen with 459 drugs to analyze subtype specific drug sensitivities and identified a robust anticancer activity of HSP90 inhibitors in the CMS1 (MSI immunogenic) and the CMS4 (mesenchymal) subtypes [32]. Both subtypes associate with an unfavorable outcome for metastatic CRC patients [7,8]. We further showed that the HSP90 inhibitor luminespib had the potential to alleviate resistance to 5-FU in a CMS4-PDX model derived from a liver metastasis of a CRC patient. In contrast, in a chemosensitive CMS2-PDX model, luminespib did show antitumor activity as monotherapy, but no synergistic effect in combination with 5-FU. Interestingly, the anticancer activity of luminespib (monotherapy or in combination with 5-FU) was target specific only in the CMS4 model, as shown by increased HSP70 expression levels [32]. Considering the selective activity *in vitro*, and the chemosensitizing activity *in vivo*, CMS1/4 subtypes might serve as predictive biomarkers for stratified treatment of HSP90 inhibitors against CRC.

6. Concluding remarks

The clinical efficacy of HSP90 inhibitors for CRC is likely to be improved with combination therapies and molecular stratification of patients. Effect of combinations with standard CRC therapies has been demonstrated in early-phase clinical trials, while clinical data for combinations with experimental therapies are pending. Immunotherapy combinations are particularly appealing, based on the rationale that HSP90 inhibition may mediate inhibition of oncogenes that promote immunosuppression. A promising aspect of chaperone inhibition is the increased production of neoantigens, due to client-proteasomal-degradation, that potentially can turn cold tumors hot. Furthermore, HSP90-inhibitors appear to have differential activity in CMS1/4; thus, upfront patient stratification of these subtypes might improve the anticancer activity of conventional chemotherapy if combined with HSP90 inhibitors. Therefore, the chances for drug resistance decrease with the application of rational drug combinations, and inhibition of HSP90 offers a wide range of opportunities to enhance the anticancer effects of other drugs.

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