In desmoid-type fibromatosis cells sorafenib induces ferroptosis and apoptosis, which are enhanced by autophagy inhibition

Anne-Rose W. Schut [a, b], Anne L.M. Vriends [a], Andrea Sacchetti [c], Milea J.M. Timbergen [a, b],
Benjamin A. Alman [d, e], Mushriq Al-Jazrawe [d, f], Dirk J. Grünhagen [b], Cornelis Verhoef [b],
Stefan Sleijfer [a], Erik A.C. Wiemer [a, *]

[a] *Department of Medical Oncology, Erasmus MC Cancer Institute, University Medical Center Rotterdam, the Netherlands*
[b] *Department of Surgical Oncology, Erasmus MC Cancer Institute, University Medical Center Rotterdam, the Netherlands*
[c] *Department of Pathology, Erasmus MC Cancer Institute, Erasmus University Medical Center, Rotterdam, the Netherlands*
[d] *Developmental & Stem Cell Biology Program, Hospital for Sick Children, Toronto, ON, Canada*
[e] *Department of Orthopaedic Surgery, Duke University, Durham, NC, USA*
[f] *Cancer Program, Broad Institute of MIT and Harvard, Cambridge, MA, USA*

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ABSTRACT

Introduction: Desmoid-type fibromatosis (DTF) is a rare, soft tissue tumour. Sorafenib, a multikinase inhibitor, has demonstrated antitumour efficacy in DTF patients. Little is known about the underlying molecular mechanisms, which are crucial to know to further optimize systemic treatments. Here we investigated the molecular effects of sorafenib exposure on DTF and stromal cells, with an emphasis on cell death mechanisms.
Material and methods: DTF primary cell cultures, with known CTNNB1 status, and primary stromal cell cultures, derived from DTF tissue, were exposed to clinically relevant concentrations of sorafenib in the presence or absence of inhibitors of ferroptosis, apoptosis and autophagy. Cell viability was determined after 24 and 48 h using MTT assays. Annexin V/PI staining, lipid peroxidation analysis and immunoblotting were performed to assess apoptosis, ferroptosis and autophagy.
Results: Exposure to sorafenib caused a significant, concentration- and time-dependent decrease in cell viability in all primary DTF and stromal cell cultures. Inhibitors of ferroptosis and apoptosis protected against sorafenib-mediated cytotoxicity implicating that both cell death mechanisms are activated. Annexin V/PI stainings and lipid peroxidation analyses confirmed induction of apoptosis and ferroptosis, respectively. Autophagy inhibition enhanced the cytotoxic effect of sorafenib and led to a stronger induction of apoptosis and ferroptosis.
Conclusion: This study identified ferroptosis and apoptosis as mechanisms for the sorafenib induced cell death in DTF cells as well as stromal cells. Furthermore, autophagy inhibition enhanced the cytotoxic effects of sorafenib. Knowledge of the mechanisms by which sorafenib affects DTF at a cellular level may help to optimize its clinical efficacy and mitigate toxic effects.

1. Introduction

Sporadic desmoid-type fibromatosis (DTF) is a rare, clonal fibroblastic proliferation of the soft tissue cells arising in musculoaponeurotic structures [1]. The estimated incidence in the population is 2—5 patients per million people per year [2,3]. DTF commonly occurs in the extremities, the abdominal wall and at intra-abdominal locations. Although they are benign tumours without metastatic potential, they can cause significant morbidity by local infiltrative growth and their tendency to recur locally [1,4]. The biological behaviour of DTF is unpredictable and variable, exhibiting stages of progressive growth, growth stabilization and even regression of the tumour without any treatment [4,5].

Desmoid tumours do occur in the context of familial adenomatous polyposis (FAP), but the majority of desmoid tumours are

* Corresponding author. Dept. of Medical Oncology, Erasmus MC Cancer Institute Erasmus Medical Center, Room Be422 Wytemaweg 80 3015 CN Rotterdam, the Netherlands.
E-mail address: e.wiemer@erasmusmc.nl (E.A.C. Wiemer).

sporadic and molecularly characterized by mutually exclusive mutations in exon 3 of the β-catenin (CTNNB1) gene [6]. The most common mutations lead to a substitution of threonine at position 41 with alanine (T41A) or cause replacement of serine at position 45 with phenylalanine (S45F) or proline (S45P). The mutations block phosphorylation of threonine or serine residues and result in stabilization of β-catenin which is subsequently translocated into the nucleus where it transcriptionally activates Wnt target genes [7]. The remainder of sporadic desmoid tumours (5—15%) are classified as wild-type (WT) as they lack mutations in CTNNB1 exon 3. It is unclear what drives the formation of these WT tumours [8]. Studies reported a potential clinical impact of the CTNNB1 mutational subtype, with S45F-mutated DTF tumours exhibiting a higher risk of recurrence after primary surgery and a different response to systemic treatment [9—12]. However, the prognostic role of the S45F mutation is a matter of discussion since others reported conflicting results and could not find an effect of CTNNB1 mutation status on outcome [9,13,14].

The unpredictable clinical course of DTF makes it challenging to choose an appropriate treatment strategy. The currently recommended first line treatment is active surveillance, while local or systemic treatment options may be considered in cases of progressive and symptomatic disease [15]. Unfortunately, these active forms of treatment do not guarantee tumour reduction or clinical benefit. The rate of recurrence after surgical resection is high: up to 60%, while there is only a modest overall response to systemic therapies such as chemotherapy [7,15—17].

Tyrosine kinase inhibitors have been suggested as a treatment option for DTF, of which sorafenib is the most promising [18]. Recent studies have demonstrated the antitumour effect of sorafenib in DTF patients [5,19]. Results of a randomized phase 3 clinical trial showed a 2-year progression-free survival of 81% for patients who received sorafenib compared with 36% in the placebo group. The objective response rate was 33% in patients that received sorafenib and 20% in the control (placebo) group [5]. Despite the observed benefit in this study, a withdrawal rate of 20% was observed due to drug-related toxic effects, emphasizing the need to determine optimal use of sorafenib in DTF patients.

Sorafenib is a multikinase inhibitor and targets several receptor tyrosine kinases such as vascular endothelial growth factor receptors (VEGFR2, VEGFR3), platelet-derived growth factor receptor beta (PDGFRB), Flt-3 and c-KIT as well as Raf kinases (B-RAF, C-RAF) [20,21]. Currently, little is known about the cellular effects of sorafenib in DTF. Knowledge of the cellular effects, particularly the cell death mechanisms that are induced, could help to identify DTF patients who may benefit from sorafenib therapy and to reduce the drug-related toxic effects or drug resistance. Studies, using cell lines derived from other tumour types than DTF, have shown that sorafenib is capable of inducing apoptosis [22—24]. It has also been reported that sorafenib can induce ferroptosis in hepatocellular carcinoma (HCC) cells [25,26]. Ferroptosis is a form of programmed cell death that is distinct from apoptosis, necrosis and autophagy, and is characterized by oxidative stress and iron-dependent accumulation of lipid hydroperoxides [27]. Some studies of HCC cells have shown that autophagy acted as protective mechanism against cytotoxic effects of sorafenib, while others described that autophagy induced by sorafenib enhanced its antitumour effect [28—30]. This ambivalent role of autophagy highlights the importance of exploring its function in response to sorafenib treatment in DTF cells.

Few studies have suggested that the cytotoxic effects of sorafenib in DTF cells are caused by inhibition of proliferation and invasion, and induction of apoptosis [31,32]. No previous study has investigated the induction of ferroptosis in DTF cells. The aim of this study is to investigate the antitumour effects of sorafenib in DTF cells. A better understanding of the underlying molecular mechanisms could help to improve the efficacy, and therefore the clinical benefits, of sorafenib therapy.

2. Material and methods

2.1. Cell cultures

The primary cell cultures D5, D7, D8, D9 and D11 were isolated from desmoid tumour tissue at the Erasmus Medical Centre Rotterdam, the Netherlands. A detailed description of the cell cultures can be found in Appendix A. The cell line AF208 was a kind gift from Dr. B. Alman (The Hospital for Sick Children, Toronto, Canada) and cultured under similar conditions. At regular intervals the cell cultures were subjected to genotyping focusing on the presence of specific CTNNB1 exon 3 mutations as previously described by Dubbink et al. [33]. The passages used for the experiments of cell lines D7, D9 were heterozygous for the S45F mutation and the cell line D8 was heterozygous for T41A mutation, thereby matching the genotype of the primary tumours. No CTNNB1 exon 3 mutations could be detected in passages of D5 and D11, whereas the corresponding primary tumours did harbour exon 3 mutations. Therefore, the cell lines D5 and D11 were considered derived from desmoid stromal cells with a wild-type CTNNB1 gene. The cell line AF208 was confirmed to be heterozygous for the S45F mutation. This study was conducted as part of an experimental protocol entitled "Translational research on soft tissue sarcomas" which was approved by the Medical Ethics Committee of the Erasmus MC, Rotterdam the Netherlands (MEC-2016-213). The use of anonymous or coded left-over material for scientific purposes is part of the standard treatment agreement with patients. Therefore, no informed consent was required according to the applicable Dutch rules and legislation.

2.2. Chemicals

Sorafenib was purchased from LC Laboratories, hydroxychloroquine sulfate from Tebu-Bio, Z-VAD-fmk from AbMole BioScience, and deferiprone (DFP) and ferrostatin-1 were obtained from Sigma-Aldrich. Stock solutions of sorafenib, Z-VAD-fmk and ferrostatin-1 were prepared in DMSO, DFP was dissolved in DMEM (Gibco) and hydroxychloroquine was dissolved in PBS.

2.3. Assays

Cell viability was assessed after 24 and 48 h of compound exposure using MTT assays. Annexin V/PI staining, lipid peroxidation analysis and immunoblotting were performed to assess apoptosis, ferroptosis and autophagy. A detailed description of the procedures is presented in Appendix A.

2.4. Statistical analysis

Differences in cell viability between the conditions were evaluated by using a Student t-test. For all statistical tests, a two-sided p-value <0.05 was considered statistically significant and marked by a single asterisk (*). P-values <0.01 were marked by double asterisks (**) and <0.001 by triple asterisks (***). All statistical tests were performed with SPSS (IBM SPSS Statistics, Version 26.0).

3. Results

3.1. Sorafenib affects viability of DTF derived tumour and stromal cells

To examine the effect of sorafenib on DTF cells, we first performed a cytotoxicity assay on four primary DTF cell cultures (D7^{S45F}, D8^{T41A}, D9^{S45F}, AF208^{S45F}) and two primary cell cultures derived from desmoid stroma (D5^{WT}, D11^{WT}). Exposure to sorafenib resulted in reduction of cell viability in all cell lines. Maximum effects were seen at a concentration of 20 μM sorafenib, resulting in a significant decrease in cell-viability after 24 (15—30%) and 48 h (50—75%) (Fig. 1). A similar trend was seen at a concentration of 10 μM sorafenib, but the effects were less profound. A concentration of 1 μM sorafenib, used in a subset of cell lines, had little effect on cell viability of DTF cells after 24 h but caused a 8—30% reduction of cell viability after 48 h (Supplementary Fig. 1). The cell lines displayed a variability in response to sorafenib. However, these differences were not related to mutational subtype or cell origin (Fig. 1).

3.2. Inhibition of autophagy augments the cytotoxic effects of sorafenib on DTF-derived tumour and stromal cells

Autophagy is an important cell survival mechanism which could mitigate the antitumour effects from sorafenib. To investigate the possible role of autophagy on the cytotoxic effects of sorafenib, cell viability of DTF cells was assessed after the cells were treated with sorafenib in combination with hydroxychloroquine. The latter compound inhibits autophagy by decreasing autophagosome-lysosome fusion [34]. The combination of 20 μM sorafenib and hydroxychloroquine led to a significant larger reduction (10—20% more reduction) of cell viability when compared to sorafenib alone. This effect was not consistent for the combination of 10 μM sorafenib and hydroxychloroquine (Fig. 2). To confirm that hydroxychloroquine indeed caused autophagy inhibition in DTF cells, Western blot analysis of LC3 was performed. Upon autophagy LC3-I is converted to the lower migrating LC3-II which associates with

autophagosomes. Inhibition of autophagy by hydroxychloroquine results in LC3-II accumulation. Our results clearly demonstrated a decreased expression of LC3-I and an increased expression of LC3-II in DTF cells after exposure to hydroxychloroquine or, another unrelated autophagy inhibitor, bafilomycin, confirming inhibition of autophagy (Supplementary Fig. 2 and Supplementary Fig. 6). These results are in agreement with the observations made by Braggio et al. using six primary DTF cultures [31].

3.3. Sorafenib induces apoptosis in DTF cells

Previously, induction of apoptosis has been suggested as one of the mechanisms underlying in the cytotoxic effects of sorafenib in DTF cells [31]. To explore the capability of sorafenib to induce apoptosis, DTF cells were treated with a combination of sorafenib and Z-VAD-fmk, a pan-caspase inhibitor [35]. Z-VAD-fmk prevented sorafenib-induced cytotoxicity in DTF cells as its application led to a significant increase in the number of viable cells (Fig. 3a). To extend these initial observations, induction of apoptosis by sorafenib in DTF cells was further evaluated by Annexin V/PI apoptosis analysis (Fig. 3b and Supplementary Fig. 3). As shown in Fig. 3b, the percentage of apoptotic cells increased after exposure to 10 and 20 μM sorafenib in all DTF cell lines, confirming induction of apoptosis. Furthermore, a combination of hydroxychloroquine and sorafenib resulted in a greater percentage of apoptotic cells when compared to sorafenib alone (Fig. 3b). Induction of apoptosis was noticed in all cell lines examined including S45F (D7, D9, AF208), T41A-mutated (D8) DTF cells and stroma derived CTNNB1 wild-type cells (D5).

3.4. Sorafenib is capable of inducing ferroptosis in DTF cells

Ferroptosis, an iron-dependent and non-apoptotic form of cell death, is characterized by the occurrence of oxidative stress and membrane lipid peroxidation [36,37]. To explore whether the cytotoxic effects of sorafenib were also partly mediated through ferroptosis in DTF cells, we first monitored cell viability after exposure to a combination of sorafenib and ferrostatin-1. Ferrostatin-1, a
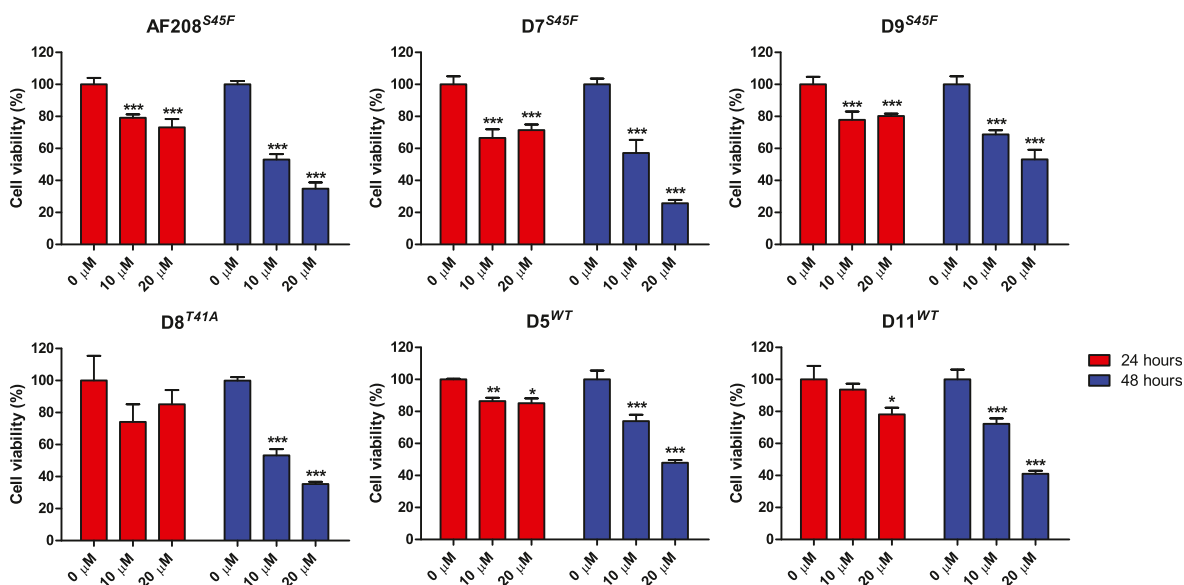

Fig. 1. Sorafenib decreases the cell viability of desmoid-type fibromatosis (DTF) and stromal cell lines. An in vitro cytotoxicity assay (MTT assay) was performed to assess the effect of sorafenib on primary DTF cell cultures and DTF-derived primary stromal cell cultures. Cells were exposed to no (0 μM; solute control), 10 μM and 20 μM of sorafenib for 24 and 48 h. Bars indicate average values ± SD (n = 3—4) *P < 0.05; **P < 0.01; ***P < 0.001 in comparison to the solute control. D7, D9 and AF208 are DTF cell lines derived from S45F-mutated desmoids; D8 is derived from a T41A-mutated desmoid tumour and D5 and D11 are cell lines derived from desmoid stroma that contain a wild-type (WT) CTNNB1.
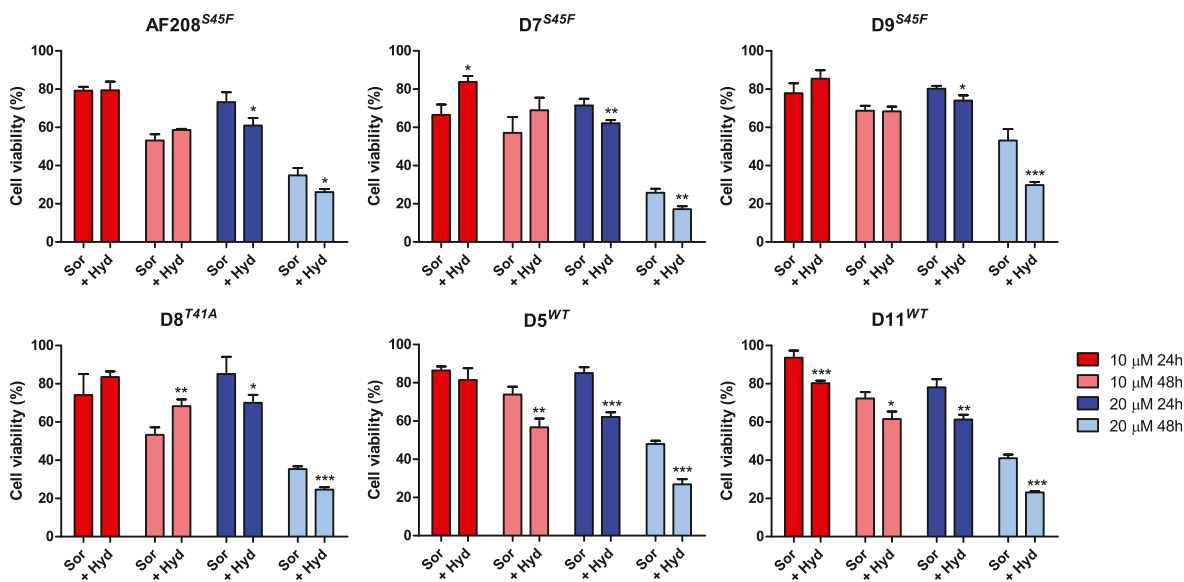
Fig. 2. Autophagy inhibition enhances the cytotoxic effect of sorafenib on desmoid-type fibromatosis (DTF) and stromal cell lines. An in vitro cytotoxicity assay (MTT assay) was performed to assess the effect of sorafenib (10 or 20 μM) with or without the autophagy inhibitor hydroxychloroquine (10 μM) on primary DTF cell cultures and DTF-derived primary stromal cell cultures. Cells were exposed for 24 and 48 h. Bars indicate average values ± SD (n = 3—4) *P < 0.05, **P < 0.01, ***P < 0.001 when compared with sorafenib alone. D7, D9 and AF208 are DTF cell lines derived from S45F-mutated desmoids; D8 is derived from a T41A-mutated desmoid tumour and D5 and D11 are cell lines derived from desmoid stroma and contain a wild-type (WT) CTNNB1. Abbreviations: Sor, sorafenib; Hyd, hydroxychloroquine.

pharmacological inhibitor of ferroptosis, inhibits lipid peroxidation by scavenging free hydroperoxyl radicals and initiating alkoxyl radicals produced by ferrous iron from lipid hydroperoxides [27,38]. We observed that ferrostatin-1 protected DTF cells from the sorafenib-induced cytotoxicity, suggesting that sorafenib can, in addition to apoptosis, also induce ferroptosis in DTF cells (Fig. 4a). As accumulation of lipid hydroperoxides is one of the hallmarks of ferroptosis, we performed a lipid peroxidation analysis using a Bodipy-C11 probe to confirm the occurrence and accumulation of lipid hydroperoxides in DTF derived cells. An increase in fluorescent signal at 510 nm of this probe reflects increased lipid peroxidation. Sorafenib slightly induced lipid peroxidation in all DTF cell lines, and 20 μM sorafenib resulted in a larger increase in fluorescence compared to 10 μM sorafenib (Fig. 4b and Supplementary Fig. 4). The addition of hydroxychloroquine further enhanced the amount of sorafenib-induced oxidative stress, and hence the induction of ferroptosis as judged from the increased fluorescent signal of the Bodipy-C11 probe in DTF derived cells at a sorafenib concentration of 10 μM. This effect was less clear when cells were exposed to 20 μM sorafenib and hydroxychloroquine (Fig. 4b and Supplementary Fig. 4). Finally, DTF cells were treated with a combination of sorafenib and DFP, an iron chelator that inhibits ferroptosis by preventing iron-dependent lipid peroxidation [39]. Application of DFP led to a significant increase in the number of viable cells (Supplementary Fig. 5). Overall, these findings indicate that sorafenib induces both apoptosis and ferroptosis in DTF-derived cells and that autophagy inhibition intensifies its cytotoxic activity.

4. Discussion

The aim of this study was to investigate the molecular effects of sorafenib exposure on DTF and stromal primary cell cultures with special attention to cell death mechanisms. It is of interest to determine sorafenib's mechanism of action in further detail to more accurately define sorafenib's targets causing its antitumour activity. The results of this study show that sorafenib is capable of inducing ferroptosis and apoptosis simultaneously in DTF and stromal cells, and that inhibition of autophagy enhances the cytotoxic effects of sorafenib.

Induction of cell death resulting in tumour stabilization or regression is one of the main goals of DTF systemic treatments. Most studies have focused on apoptosis as mechanism of action, but more recently non-apoptotic forms of cell death, particularly ferroptosis, received much interest [25,36,37,40,41]. Some studies have previously shown that sorafenib is able to induce ferroptosis in hepatocellular carcinoma cells and different solid tumours, most likely by functioning as a competitive inhibitor of the system X_c^- cystine/glutamate antiporter thereby affecting glutathione synthesis [25,26,41,42]. In contrast to these findings, however, Zheng et al. recently reported that sorafenib is not able to induce ferroptosis in various, predominantly epithelial, cancer cell lines [43]. In these experiments sorafenib was compared to erastin and sulfasalazine both potent inhibitors of system X_c^- and it is claimed that sorafenib is a poor inhibitor of system X_c^-. A confounding factor interpreting these experiments is the fact that sorafenib also induces apoptosis which may obscure ferroptosis induction. Our findings, using DTF cells do indicate that sorafenib exposure does induce ferroptosis to some extent (as well as apoptosis). In this context it should be noted that DTF cells are mesenchymal in origin and it has been observed that especially the mesenchymal cell state is dependent on a lipid peroxidase pathway, in which glutathione peroxidase 4 (GPX4) plays a central role, that protects against ferroptosis [44,45]. Hence, mesenchymal cells are prone to execute ferroptosis when GPX4 is inhibited.

To the best of our knowledge, this is the first study exploring the role of ferroptosis in DTF cells. Our current study found that sorafenib-induced cell death was significantly inhibited by the ferroptosis inhibitors ferrostatin-1 and DFP and that exposure to sorafenib led to lipid related oxidative stress in DTF cells. Together, these results support the conclusion that sorafenib is capable of inducing ferroptosis in DTF cells. Interestingly, Dixon et al. [42] and Lachaier et al. [25] reported that none of the other kinase inhibitors that target a subset of kinases comparable to sorafenib, such as imatinib, were able to induce ferroptosis in human kidney
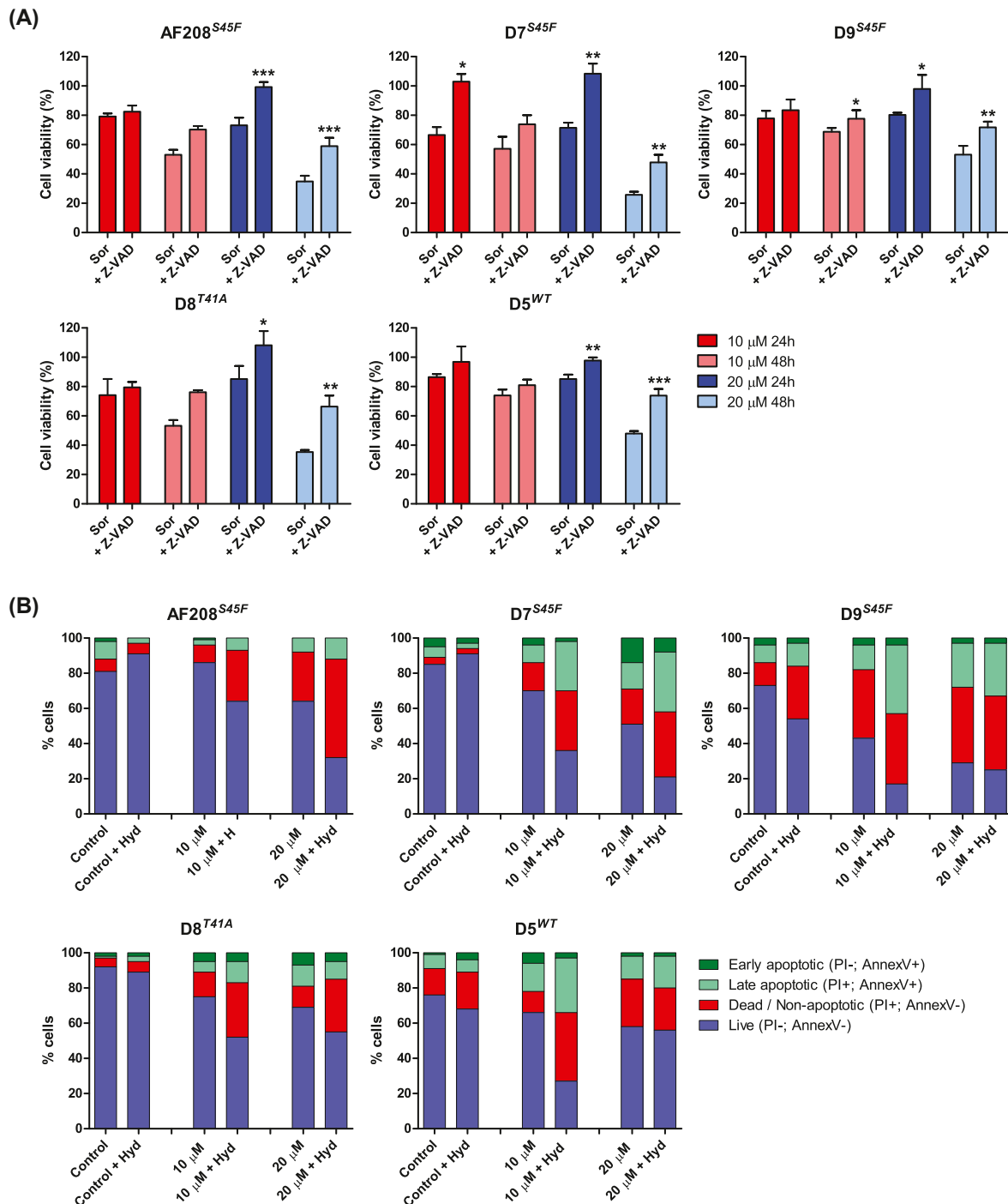
(A)

(B)

Fig. 3. Sorafenib induces apoptosis in desmoid-type fibromatosis (DTF) cells. (A) Cytotoxicity of sorafenib in DTF-derived cell lines was determined after exposure to sorafenib (10 and 20 μM) with or without Z-VAD-fmk (20 μM) for 24 and 48 h using an MTT assay. Bars indicate average values $\pm$ SD (n = 3–4) *P < 0.05, **P < 0.01, ***P < 0.001 when compared with sorafenib alone. (B) Annexin V/PI apoptosis analysis of DTF-derived cell lines after exposure to sorafenib (10 and 20 μM) with or without hydroxychloroquine (10 μM) for 24 h. Each bar is based on the FACS analysis of 20,000 events. D7, D9 and AF208 are DTF cell lines derived from S45F-mutated desmoids; D8 is derived from a T41A-mutated desmoid and D5 is derived from desmoid stroma and contains a wild-type (WT) CTNNB1. Abbreviations: Sor, sorafenib; Z-VAD, Z-VAD-fmk; Hyd, hydroxychloroquine; PI-/+, propidium iodide negative/positive; AnnexV-/+, Annexin V negative/positive.

cancer and colon carcinoma cells; this making ferroptosis induction a unique property of sorafenib. It may be of interest to evaluate whether the capability to induce ferroptosis has influence on clinical response, as the response to sorafenib in DTF patients appears to be more pronounced than to imatinib [15]. Serum level of advanced oxidation protein products (AOPP) could potentially be used to evaluate effectiveness of sorafenib in DTF patients, as a

study by Coriat et al. showed that an increased serum level AOPP was correlated with the clinical response in HCC patients [46]. Likewise, it would be important to assess whether ferroptosis plays a role in the potential development of toxic effects after sorafenib treatment to determine optimal clinical use. Furthermore, other ferroptosis inducers may present with more tolerable toxicities than sorafenib.
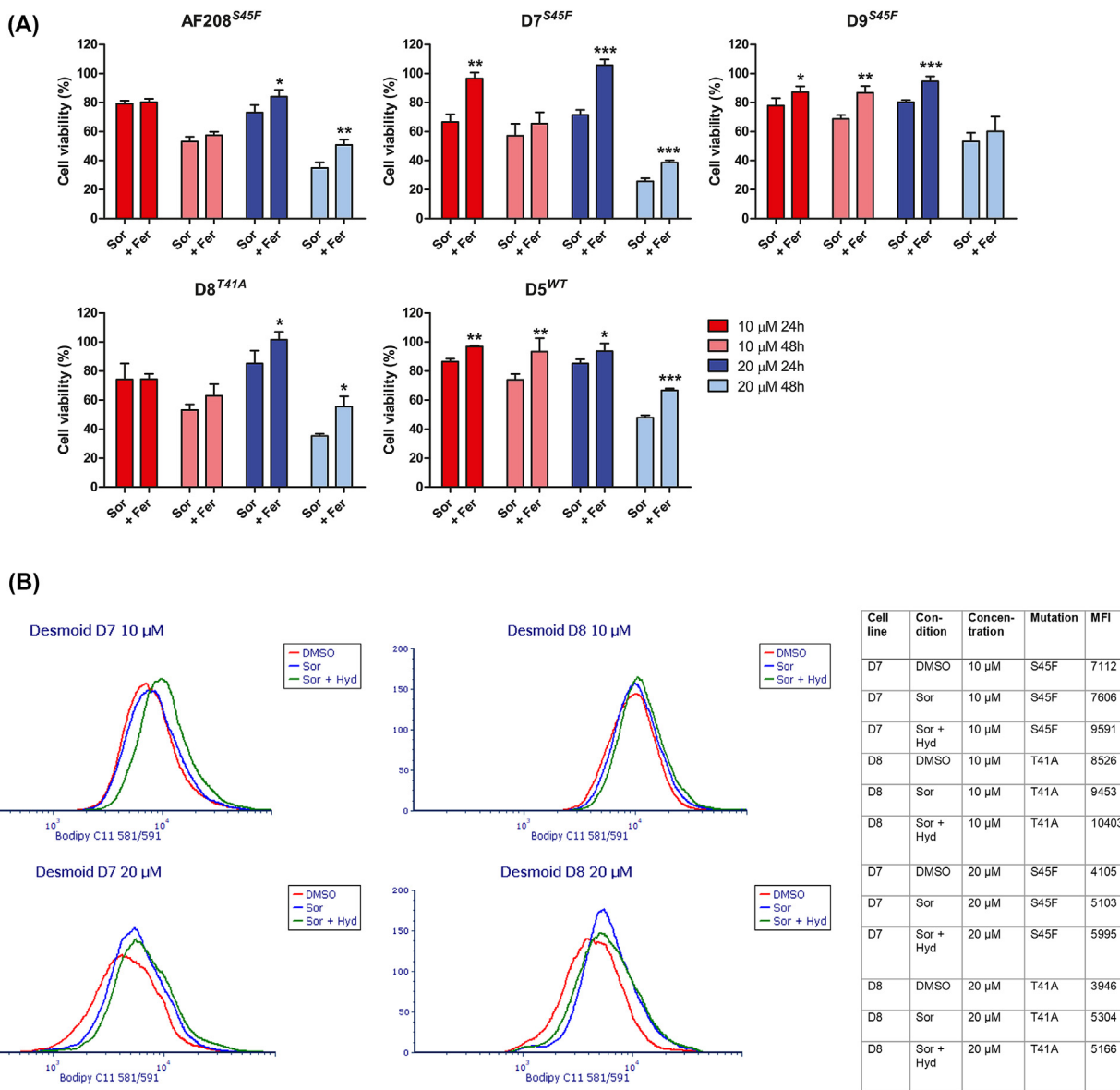
Fig. 4. Sorafenib induces ferroptosis in DTF cells. (A) Cytotoxicity of sorafenib in DTF derived cell lines was determined after exposure to sorafenib (10 and 20 μM) with or without ferrostatin-1 (1 μM) for 24 and 48 h using an MTT assay. Bars indicate average values ± SD (n = 3–4) *P < 0.05, **P < 0.01, ***P < 0.001 when compared with sorafenib alone. (B) Lipid ROS assay analysing the Bodipy-C11 signal intensity of DTF cells (D7 and D8) after exposure to sorafenib (10 and 20 μM) with or without hydroxychloroquine for 16 h. An increase in fluorescent signal of this probe reflects increased lipid peroxidation. The mean fluorescence intensity of the Bodipy-C11 probe is depicted in the table on the right. The graphs are based on the FACS analysis of 20,000 events. D7, D9 and AF208 are DTF cell lines derived from S45F-mutated desmoids; D8 is derived from a T41A-mutated desmoid and D5 is derived from desmoid stroma and contains a wild-type (WT) CTNNB1. Abbreviations: Sor, sorafenib; Hyd, hydroxychloroquine; Fer, ferrostatin-1; MFI: Mean fluorescence intensity; DMSO, dimethylsulfoxide.

Because apoptosis has previously been identified as a mechanism of cell death induced by sorafenib therapy, the current study also evaluated the induction of apoptosis by sorafenib in DTF cells [26,31,42]. The application of the caspase inhibitor Z-VAD-fmk was found to - at least partially - protect DTF cells from the cytotoxic effects of sorafenib, and exposure to sorafenib resulted in an increase of the percentage of apoptotic cells. Thus, next to ferroptosis, these results indicate that sorafenib can induce apoptotic cell death in DTF cells. The two cell death mechanisms appear to occur parallel to each other and their relative contribution to DTF cell viability may change in time. We observed that both Z-VAD-fmk and ferrostatin-1 failed to protect DTF cells completely after 48 h, suggesting that when one of the mechanisms is blocked, sorafenib is able to exert its cytotoxic effect through the other mechanism.

Autophagy is a catabolic process which uses the lysosomal system for degradation of damaged or useless proteins and organelles and is essential for maintaining cellular homeostasis [47,48]. This mechanism has an important role in cancer development, as it could promote either tumour suppression or tumour progression [49,50]. In response to drug exposure, autophagy can lead to cell death or cell survival of tumour cells, contributing to drug responsiveness or resistance [47,48,51]. The current study demonstrated that inhibition of autophagy by hydroxychloroquine enhanced the cytotoxic effects of sorafenib in all DTF cell lines. Furthermore, a stronger induction of apoptotic cell death was achieved after exposure to a combination of sorafenib and

hydroxychloroquine. This finding in HCC cells was also reported by Shimizu et al. [29], who found that autophagy inhibition by hydroxychloroquine increased apoptosis after sorafenib treatment. These results may be explained by the crosstalk between apoptosis and autophagy, as it has been suggested that in many cases inhibition of autophagy could lead to apoptosis induction, and vice versa [51]. Looking at the interaction between autophagy and ferroptosis, previous studies have shown inconsistent results. While some studies reported that autophagy promoted the induction of ferroptosis [52—54], Zhao et al. demonstrated that autophagy protected cells from ferroptotic cell death [55]. The latter conclusion accords with our observations, which showed stronger ferroptosis induction after addition of hydroxychloroquine. This effect was more profound at 10 μM sorafenib than at 20 μM sorafenib. A possible explanation for this might be that a higher concentration of sorafenib could outcompete the protective effect of autophagy and consequently induce ferroptosis. Further research is clearly needed to better understand the effect of autophagy inhibition on induction of ferroptosis and apoptosis by sorafenib in DTF cells. The finding that autophagy inhibition enhanced the cytotoxic effects of sorafenib may be promising to improve clinical efficacy of sorafenib in DTF patients, either by increasing effectiveness of sorafenib or by enabling the clinician to reduce sorafenib dosage (with the accompanying less toxicity) but the same effect. Phase I and phase II clinical studies evaluating the efficacy of the combination of sorafenib and hydroxychloroquine in refractory or relapsed solid tumours [56] and hepatocellular cancer [57] are ongoing and their preliminary data is encouraging [58]. Results of these studies could potentially be used to guide a future clinical trial evaluating the effectiveness of sorafenib and hydroxychloroquine in DTF patients.

The exact molecules with which sorafenib interacts in DTF cells, be it kinases or other biomolecules, are not known. As sorafenib is a multikinase inhibitor with multiple targets, other mechanisms, such as anti-angiogenic effects, may also contribute to the antitumour effects of sorafenib in DTF. Furthermore, sorafenib may have antitumour effects in DTF trough inhibition of the ERK and PI3K/Akt/mTor signaling pathway [31,32]. While our study only evaluated direct effects of sorafenib in DTF cells, these effects on several receptor tyrosine kinases may also contribute to response of sorafenib in DTF and could potentially interfere with the induction of ferroptosis, apoptosis and autophagy. Knowledge of the downstream effects and interaction between pathways could provide options for combination therapies to overcome drug resistance or reduce toxicity.

It has been suggested that CTNNB1 mutational status could influence induction of apoptosis and response to sorafenib in DTF cells [11,31]. Braggio et al. showed that 1 μM sorafenib decreased cell viability of all DTF cell strains, but was only able to induce apoptosis in the T41A-mutated DTF cells. Combination of sorafenib and hydroxychloroquine resulted in a larger increase of apoptosis induction in the S45-mutated DTF cells [31]. Altogether, Braggio et al. concluded that response to sorafenib may differ between S45F and T41A-mutated DTF cells. This is in contrast to our findings presented here. While variability in response to sorafenib was observed between DTF cell lines, no differences in response were found between either S45F or T41A-mutated DTF cell lines. These conflicting results could be explained by differences in concentration and duration of sorafenib exposure. In the study by Braggio et al. [31], the concentration of sorafenib was low (1 μM) and cell death and apoptosis were measured at relatively late time points, varying from 3 to 7 days. Here, we measured cell viability after 24 and 48 h and found that the cytotoxic effect of sorafenib mainly is exerted at concentrations above 10 μM, which encompasses the range of the maximum plasma concentration of sorafenib (5.2—21 μM) that could

be achieved by the clinically recommended daily dose (400 mg) [42,59]. These results could indicate that a higher dose of sorafenib is required in S45F-mutated DTF cells to achieve a cytotoxic effect; however this difference seems no longer clinically relevant at concentrations above 10 μM. Knowledge of the mutation status and clinical response of patients who received sorafenib in the randomized phase 3 clinical trial [5], may help us to understand the influence of CTNNB1 mutational status on clinical effectiveness of sorafenib. Finally, we noted that the response to sorafenib of primary DTF-derived stromal cell lines, is virtually indistinguishable from that of the CTNNB1 mutated DTF lines, which may indicate that sorafenib also acts on the DTF stromal compartment.

This study has several limitations. First, our study was limited by the small number of cell lines. Moreover, our study consisted of a relatively large number of S45F-mutated DTF cell lines, whereas S45F mutations usually represent about 20% all DTF [9,10]. The limited availability of DTF cell lines is partly due to the rarity of DTF, but also to the fact that there is less DTF tissue available as active surveillance is the currently recommended first line treatment strategy [15]. Future studies consisting of more cell lines harbouring the different mutational subtypes are needed to confirm our results and to determine the association between CTNNB1 mutation status and response to sorafenib in DTF patients. In addition to cell line experiments, in vivo studies of biopsies taken from DTF tumours can contribute to the assessment of the effects of sorafenib exposure in DTF patients and may be performed in the future.

5. Conclusions

This study identified both ferroptosis and apoptosis as mechanisms for the sorafenib induced cell death in DTF cells as well as DTF-derived stromal cells. Furthermore, autophagy inhibition enhanced the cytotoxic effects of sorafenib. Knowledge of the molecular mechanisms by which sorafenib affects DTF at a cellular level may help to optimize its clinical efficacy and mitigate toxic effects. Further research is required to more precisely establish the role and contribution of ferroptosis, apoptosis and the involvement of autophagy in the clinical response and to determine the value of CTNNB1 mutation status in response to sorafenib.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author.

CRediT authorship contribution statement

Anne-Rose W. Schut: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing — original draft, Writing — review & editing. **Anne L.M. Vriends:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing — original draft, Writing — review & editing. **Andrea Sacchetti:** Formal analysis, Writing — review & editing. **Milea J.M. Timbergen:** Resources, Writing — review & editing. **Benjamin A. Alman:** Resources, Writing — review & editing. **Mushriq Al-Jazrawe:** Resources, Writing — review & editing. **Dirk J. Grünhagen:** Conceptualization, Writing — review & editing. **Cornelis Verhoef:** Conceptualization, Writing — review & editing. **Stefan Sleijfer:** Conceptualization, Funding acquisition, Investigation, Project administration, Writing — review & editing.

Erik A.C. Wiemer: Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Visualization, Supervision, Writing − original draft, Writing − review & editing, All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

None.

Acknowledgments

Not applicable.

Appendix A. Supplementary data

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