Combination of cytotoxic agents and targeted therapy for the treatment of advanced sarcomas: preclinical background and early clinical development

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Y seguro que no hay dios que le siga el ritmo…

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1. Introduction

Sarcomas are a group of diseases that account for around 1% of human malignancies (1). The number of new cases of sarcoma per year ranges from 2.4 to 3.6 per 100,000 people (2) and no curative treatment is currently available in the advanced setting. This constitutes a health problem that needs to be addressed.

The broad term "sarcoma" comprises more than 50 distinct malignant neoplasms recognized by the World Health Organization (WHO) classification of tumors (3). Despite their variety, they share the common feature of arising from the embryonic mesoderm. Nevertheless, it is still not fully clear whether they originate from different committed cell types or from a multipotent cell subsequently driven into a certain lineage (4). Whichever the cell of origin, it is known that some hereditary conditions such as the Li-Fraumeni (5) syndrome are predisposing factors for developing sarcomas. However, they are sporadic tumors in the majority of cases. Genetically, they can be divided in 2 big groups according to their underlying aberrations: sarcomas with complex karyotypes and sarcomas with specific associated chromosomal translocations. The latter generate hybrid oncogenes that often code for transcription regulators leading to oncoproteins involved in sarcomagenesis (6, 7).

Sarcomas can affect patients of all ages although certain specific subtypes such as Ewing's sarcoma are more frequent in children and young adults (1). Also, given the ubiquity of the mesenchymal tissue, almost every part of the body may be involved. Generally speaking, sarcomas are classified in soft tissue or bone sarcomas depending on the tissue of origin. Soft tissue sarcomas are the most common subgroup, with leiomyosarcoma, synovial sarcoma and liposarcoma being the most frequent (3). Bone

sarcomas are rarer and they are only around 20% of all sarcomas. Osteosarcoma and Ewing's sarcoma are the most reported types within this subset and they tend to appear at younger ages (1).

In spite of their very distinct characteristics, the prognosis of patients affected by sarcomas is generally poor and it has not improved significantly in recent years (8, 9). Around half of all patients diagnosed with sarcoma will eventually develop metastases at some point in the course of their disease even if they are diagnosed at an early stage (8). This aggressive behaviour, together with the poor results achieved with the drugs currently available, result in a median overall survival (OS) of around 1 year in the advanced setting and a 5-year survival rate of 15% (9). For patients with local relapse, the disease specific survival is lower than 50% at 5 years (10). With such disappointing figures, it is therefore mandatory to identify new active therapeutic strategies able to improve the outcome of patients affected by this group of diseases. A number of efforts have been made in last decades but no major successes have been achieved (11). In recent years, a series of novel targeted drugs have been developed in oncology (12) but, unfortunately, only a few of those new compounds have achieved positive results in sarcomas, gastro-intestinal stromal tumor (GIST) being the most important example (13, 14). Thus, only 2 phase III trials with new targeted therapies in sarcomas other than GIST have been reported as positive to date (one with the antiangiogenic agent pazopanib and the other with the mammalian target of rapamycin (mTOR) inhibitor ridaforolimus), indicating that further investigation is needed (15, 16). Moreover, the revolution of immunotherapy in cancer treatment that we are currently witnessing in a number of tumors has still not affected sarcomas. With the exception of the affinity enhanced T-cell therapy targeting NY-ESO antigen in synovial sarcoma

(17), immunotherapeutic agents have not achieved clinically meaningful success in sarcomas to date (18). One of issues that has classically made difficult the finding of novel active drugs in these malignancies is the absence of molecular therapeutic targets. However, great advances have been recently made in the understanding of sarcomas' molecular biology (19) and their relevant cellular pathways with their key effectors (20). For instance, angiogenesis or the mTOR signaling pathway, among others, have been described to have great importance in sarcoma pathogenesis (21, 22). The development of therapeutic strategies that inhibit such pathways, in combination with active cytotoxic agents able to enhance their efficacy without significant toxicity, is therefore worth exploring.

2. Active cytotoxic drugs in sarcomas: ifosfamide and gemcitabine

2.1 Ifosfamide

Ifosfamide and doxorubicin are the most active drugs in sarcomas to date. However, they achieve response rates (RR) of only 20−30% or even lower depending on the series (23, 24). Both compounds have been used for decades for the treatment of sarcomas. Several randomized studies have compared doxorubicin or ifosfamide with other agents but no treatment has yet demonstrated to be superior to these 2 classic cytotoxic agents (18). Subsequently, they remain as the standard of care in early lines of sarcoma treatment (25).

Ifosfamide is a cytotoxic alkalizing agent belonging to the oxazaphosporine class of compounds. Its mechanism of action is by inducing DNA cross-links, blocking tumor cells in late S phase and early G2 phase of the cell cycle (26). As single agent, ifosfamide is usually given in second line of treatment in the advanced setting after failure to doxorubicin (25). When compared to doxorubicin, efficacy results of ifosfamide are similar but when it is used in second line, responses are as low as 5-8%. Median OS of patients treated with ifosfamide after failure to doxorubicin is 35-45 weeks and the median time to progression is just 6-14 weeks (24). Higher RR has been achieved with concomitant administration of both doxorubicin and ifosfamide but this regimen is not exempt from potentially serious toxicity, limiting its use to a selected population of very fit patients (27). Unfortunately, this is not the most common situation in real-life clinical practice so the use of this combinatory schedule is not generalized.

Ifosfamide activity and toxicity is related with the dose (28). The most commonly used schedule is 3 g/m2 administered on days 1, 2 and 3, every 3 weeks (24). Ifosfamide profile of adverse events include haemorrhagic cystitis, myelotoxicity, nephrotoxicity and neurotoxicity (18). Cystitis secondary to ifosfamide is caused by its metabolite acrolein, which can lead to haemorrhagic cystitis and dysuria. Concomitant administration of sodium 2-mercaptoethane sulfonate (mesna) prevents and treats such toxicity and it is routinely included in ifosfamide treatment schemes (29). The other most important side effect of ifosfamide, neurotoxicity usually in the form of encephalopathy, occurs in up to 10% of all treated patients or even more depending

on the schedule (30). It is treated with intravenous methylene blue and it can be fully reversed in the majority of cases. On the other hand, renal toxicity induced by ifosfamide is potentially irreversible and cumulative. It is prevented by aggressive hydration during the ifosfamide infusion (26).

Overall, ifosfamide is considered a reasonably safe treatment for sarcoma patients since its most serious adverse events can be prevented or treated with well-known and easy measures such as the ones already mentioned. Combinatory regimes including ifosfamide are therefore promising in terms of both activity and safety.

2.2 Gemcitabine

Gemcitabine is an antimetabolite agent that has also demonstrated to be active in sarcomas. It is a nucleosid analog where two fluorine atoms have replaced the hydroxyl on the ribose. Its antiproliferative action is exerted after tumoral conversion into active triphosphorylated nucleotides interfering with DNA synthesis and targeting ribonucleotide reductase (31). Up to 6 single arm phase II studies have evaluated gemcitabine alone in sarcomas patients who progressed to doxorubicin (32-37). Median OS range from 7.2 to 13.9 months whereas the RRs reported varied from 3.1% to 17.9%. This wide variability can be explained by the different schedules used in the trials: gemcitabine was administered over 30 minutes in 3 studies, over 100 minutes in 2 studies and over 360 minutes in 1 study, with planned dose-intensity spanning from 200 mg/m2/week to 830 mg/m2/week (38). Overall, these results show that although gemcitabine is an active treatment in sarcomas, its efficacy as single agent is modest.

Combinations of gemcitabine with other cytotoxic drugs after failure to doxorubicin have also been evaluated in several clinical trials. In general, they achieved good efficacy results with a tolerable profile of side effects (37, 39, 40). For instance, a comparative randomized phase II study assessed gemcitabine alone vs gemcitabine plus docetaxel in leiomysarcoma patients stratified by uterine or non-uterine origin. Results did not show significant differences in terms of response between the monotherapy and the 2-drug arms (37). Similar gemcitabine plus docetaxel schedule was assessed in 3 phase II trials in patients with leiomyosarcoma. In the uterine origin populations, response rates reported were 52%, 25% and 24%, with a tolerable profile of side effects (37, 41, 42). However, the best RR reported in the non-uterine origin was only 5% (37). The same combination in the same setting but using lower doses of docetaxel in Japanese population showed response in 3 out of 10 patients, with mean progression-free survival (PFS) and OS being 5.4 and 14.0 months, respectively (43). The combination of gemcitabine plus dacarbazine has also been assessed after doxorubicin treatment in 2 phase II studies. In the first study, 3-month PFS rate was 46% and in the second study it was 56% (40, 44). Furthermore, the second study - conducted by our group- showed an OS rate of 16.8 months which is clearly superior to the 8.2 months achieved by dacarbazine alone in the monotherapy arm of the trial (40).

In addition, the efficacy and toxicity of gemcitabine-based regimes, either alone or in combination, have been assessed in first line in a number of clinical trials. While single

agent gemcitabine trials yielded low RRs (from 4% to 6.3%) (45-47), results were better in combinatory schedules including docetaxel (RRs ranging from 31.5% to 35.8%) (48, 49). Maki et al. assessed the activity of gemcitabine alone versus gemcitabine plus docetaxel in both chemo-naïve and pre-treated soft tissue sarcomas patients. RRs were 16% vs 8% and median PFS was 6.2 months vs 3.0 months for the combination arm and the monotherapy arm, respectively. Median OS was also superior for the combination (17.9 months vs 11.5 months) (39). Also in first and/or second line of treatment for advanced disease, the combination of gemcitabine and vinorelbine has been evaluated in a phase II study. Results showed clinical benefit in 25% of patients with acceptable toxicity (50). Interestingly, the only phase III trial to date comparing doxorubicin vs gemcitabine plus docetaxel in first line of treatment showed superiority of doxorubicin (51).

Results from these clinical trials, together with a study with gemcitabine plus docetaxel in the adjuvant setting (52) and 3 studies with these 2 drugs including bone sarcomas (53-55), demonstrate that gemcitabine-based regimes are active and well-tolerated. Therefore, gemcitabine can be considered a suitable backbone for novel combination strategies in sarcomas (38).

3. Targeting key cellular pathways in sarcomas

3.1 Angiogenesis

Angiogenesis, the formation of new blood vessels, is one of the hallmarks of cancer (56). The formation of new blood vessels is necessary for tumor growth. Indeed, tumors cannot progress beyond about 200μm without angiogenesis (57). The uncontrolled cell proliferation driven by the carcinogenesis processes leads to a growth in the tumor mass for which an appropriate supply of oxygen and nutrients is necessary. If that supply fails, distant tumor cells undergo apoptosis or necrosis and further tumor growth is impaired. However, cancer cells are able to overcome this problem by inducing the formation of new blood vessels from pre-existing normal blood vessels in a complex multi-step process. In addition, angiogenesis not only facilitates local tumor growth but also provides a route for cancer cells to reach distant sites via bloodstream and subsequently develop metastases (58). This induction of tumour vasculature was termed the 'angiogenic switch' in the late eighties (59). It is controlled by changes in the fine-tuned balance between pro- and anti-angiogenic factors that are induced by both tumor and microenvironment cells (60). In recent years, the deep understanding of some of the complex interactions among these factors has permitted the successful development of a number of inhibitors of this important process in carcinogenesis (61). In malignancies such as clear cell renal carcinoma, in which chemotherapy and radiotherapy have classically failed in achieving meaningful activity, antiangiogenic agents have revolutionised its treatment and they are currently considered the standard of care (62).

3.1.1 Rationale for the antiangiogenic approach in sarcomas

Sarcomas are not an exception to the general tumor angiogenesis principles. However, the pattern of angiogenesis has been described to be different between sarcomas and carcinomas (63). Thus, a study found that capillaries in carcinomas are clustered in bursts within the tumor stroma and that the microvessel density in these bursts can be used as a prognostic factor whereas microvessel density in sarcomas was shown to have a more homogeneous appearance (64). Another study confirmed this observation by showing that hotspots of angiogenesis are diffuse in high-grade soft tissue sarcomas and they were present in only 33% of the investigated sarcoma specimens (65).

Some of the angiogenesis key players in cancer, such as the vascular endothelial growth factor (VEGF) and the three different forms of its receptor (VEGFR-1, VEGFR-2 and VEGFR-3), have been described to be relevant in sarcomas. For instance, correlation between serum levels of VEGF and clinical outcome in sarcoma patients has been assessed. An extensive study included 273 sarcoma patients and in 68 of them (24.9%) a significant overexpression of VEGF was found. The subtypes in which a higher expression of VEGF was detected were malignant fibrous histiocytoma (30%), carcinosarcoma (30%), leiomyosarcoma (25%) and dermatofibrosarcoma (20%). Interestingly, overexpression of VEGF had prognostic value only in leiomyosarcoma, with those patients having significantly shorter survival (66). Another study determined VEGF serum levels preoperatively in 85 patients affected by soft tissue sarcomas with the aim of assessing the relationship between VEGF and tumor grade. The poorest differentiated tumors were found to have the highest VEGF levels, proposing VEGF in serum as a biomarker of tumor aggressiveness (67). Similar results

were observed in a larger prospective series of 144 patients in whom serum levels of VEGF were not only found to have significant correlation with grade, but also with response to treatment (68). Another study further supported these findings: VEGF overexpression in paraffin-embedded tissue of surgical specimens from 79 patients with sarcomas also correlated with high tumor grade. Moreover, 78% of patients who died of disease (29 out of 37) had high VEGF expression but VEGF was not found to be an independent predictor for either OS or disease-free survival (DFS) (69). However, a similar study reported different results. VEGF expression was analyzed by immunohistochemistry (IHC) in tissue from 27 cases of thoracic sarcomas and it was found that DFS in patients with high tumor VEGF expression was significantly poorer compared to those with lower VEGF expression (70). On the other hand, it is well known that the interaction between VEGF and VEGFR-2 is probably the most crucial step in the process of angiogenesis (71). Nevertheless, it is VEGFR-3 which has been proposed to be a prognostic factor in sarcomas. In a study conducted in tissue microarrays from tumor samples of 249 patients with sarcoma in which the expression of several angiogenesis effectors was determined by IHC, high expression of VEGFR-3 was found to be an independent significant negative prognostic marker for disease-specific survival in the multivariate analysis (72). Another important player in angiogenesis is hypoxia-inducible factor 1α (HIF-1α) since this transcription factor acts as an upstream regulator of VEGF. Its importance in sarcomas was determined by assessing its expression by IHC in 49 tumor specimens. The statistical correlation with outcome showed that patients with a strong or moderate expression of HIF-1α had poorer OS than those with weak or negative expression (73). On the other hand, endoglin, a vessel marker expressed in activated endothelium, was assessed in Ewing

sarcoma and it was found to be significantly associated with worse survival (74). All these studies paved the way to consider the antiangiogenic approach as a therapeutic strategy worth exploring in sarcomas.

3.1.2 Antiangiogenic drugs in sarcomas: clinical experience

3.1.2.1 Bevacizumab

One of the first antiangiogenic agents to be clinically developed was bevacizumab. It is a humanized monoclonal antibody targeting VEGF (75) which has been assessed in combination with other drugs in several clinical studies in sarcomas. A phase II trial evaluated the addition of bevacizumab to the standard first line doxorubicin. Two out of 17 patients treated (12%) achieved partial response (PR) and 11 patients (65%) achieved stable disease (SD) for four cycles or more. Interestingly, toxicity was high: 6 patients developed cardiotoxicity grade ≥2 despite the use of the cardioprotective drug dexrazoxane when the total dose of doxorubicin exceeded 300 mg/m2 and 1 patient died of bilateral pneumothorax. These data indicates that, although the combination has clinical activity, safer schedules must be explored (76). Bevacizumab was also assessed in combination with temozolomide in a retrospective study focused on 2 specific sarcoma subtypes: hemangiopericytoma and malignant solitary fibrous tumor (hemangiopericytoma outside the central nervous system). A total of 11 out of 14 patients (79%) had PR by Choi criteria (77) and median PFS observed was 9.7 months (78). Another regimen with bevacizumab tested in sarcomas was the combination with docetaxel and gemcitabine. In this phase Ib study, 9 previously untreated patients received docetaxel, gemcitabine and bevacizumab in 3 dose-

escalating cohorts and 27 within an expansion cohort. The overall RR reported was 31.4%, including 5 complete responses (CR). However, the authors concluded that, although this is a safe and effective regimen, the benefit of adding bevacizumab to gemcitabine and docetaxel is unclear (79).

Bevacizumab has not only been studied in combination with other drugs, but also concomitant with radiotherapy as a radiosensitizer. Thus, a phase II trial enrolled 20 patients with localized soft tissue sarcomas with high risk of recurrence who received neoadjuvant bevacizumab alone followed by bevacizumab together with RT prior to the surgical resection. Pathologic necrosis of ≥80% was observed in 9 tumors (45%) and 3 more patients had a pathologic CR, which are results clearly superior to historical data with radiotherapy alone (80).

3.1.2.2 Sunitinib

Sunitinib, a multitargeted tyrosine-kinase inhibitor (TKI) which targets a number of angiogenic receptors such as VEGFR-1, VEGFR-2, VEGFR-3 and PDGF receptors (PDGFRs), among others (81), has also been studied in sarcomas. Continuous daily dose of sunitinib was evaluated in 53 advanced sarcoma patients. Ten patients (20%) experienced SD and one patient with desmoplastic small round cell tumor achieved a durable PR. Metabolic response by 18-fluorodeoxyglucose positron emission tomography (PET) was also assessed in 24 patients and, interestingly, PR and SD was seen in ten and 11 cases, respectively (82). In another study limited to 3 specific sarcoma subtypes (liposarcoma, leiomyosarcoma and malignant fibrous histiocytoma), sunitinib also showed signs of efficacy even though a considerable number of patients

enrolled in the study had been heavily pretreated. Thus, 3-month PFS rates were 75% and 69% for untreated and pretreated liposarcoma patients, respectively; 60% and 62% for untreated and pretreated leiomyosarcoma patients, respectively; and 25% and 44% for untreated and pretreated malignant fibrous histiocytoma patients, respectively (83). Other sarcoma subtypes have also demonstrated to be sensitive to sunitinib. For instance, from a total of 9 patients affected by alveolar soft-part sarcoma, a type of sarcoma characterized by its resistance to chemotherapy, 5 achieved PR, 3 had SD and median PFS was found to be 17 months (84). In solitary fibrous tumor, another sarcoma classically considered non-responsive to chemotherapy, sunitinib also showed signs of efficacy: PR by Choi criteria (77) was observed in 14 out of 35 patients whereas 16 patients achieved SD by Response Evaluation Criteria in Solid Tumors (RECIST) (85) and median PFS was 6 months (86).

In contrast to all these promising data, a phase II study by the Gynecologic Oncology Group failed to demonstrate sunitinib activity in advanced uterine leiomyosarcoma: only 2 out of 25 patients enrolled in this trial achieved PR. Furthermore, median PFS was 1.5 months and only 4 patients remained progression-free at 6 months (87).

3.1.2.3 Sorafenib

Another multi-TKI drug with antiangiogenic properties which has been found to be active in sarcomas is sorafenib. A phase II clinical trial reported in 2009 included 145 advanced sarcoma patients treated with sorafenib. Stratification was done according to the histological subtype and angiosarcoma was the only group that met the RR primary endpoint, with PR seen in 14% of 37 patients. Overall for tumor types, median

PFS was 3.2 months and median OS 14.3 months (88). However, another study focused on angiosarcoma suggested limited activity: from a total of 41 patients, no response was observed in first line while in pre-treated patients the RR was 23% (89). In a different study with sorafenib stratified by histology, the cohort that had higher median PFS was vascular sarcomas (5 months) compared to high-grade liposarcomas (2 months) and leiomyosarcomas (3 months) (90). Finally, a randomized discontinuation trial conducted across a broad range of tumor types showed activity of sorafenib in the sarcoma cohort. In this study, all patients began on open-label treatment and after 12 weeks patients with ≥25% tumor shrinkage continued on sorafenib, patients with ≤25% tumor growth discontinued treatment, and the remaining patients were randomized to receive either sorafenib or placebo. PFR after 12 weeks of treatment in the 26 patients from the sarcoma group was 31% and, overall, 1 patient achieved PR and 3 further patients achieved minor responses (91).

The therapeutic strategy of combining sorafenib with cytotoxic agents has also been evaluated. In a series of 17 sarcoma patients treated with sorafenib and the chemotherapy drug dacarbazine, 3 PR (21%) were seen as well as 6 SD (43%) with no significant toxicity, supporting sorafenib for combination regimens (92).

3.1.2.4 Pazopanib

The antiangiogenic agent which has had the more extensive clinical development in sarcomas is pazopanib. Pazopanib is a new TKI with potent antiangiogenic effects due to its affinity for VEGFR-1, VEGFR-2 and VEGFR-3 (93). Signs of activity in sarcomas were observed in a phase II trial that enrolled 142 patients with advanced sarcomas

who received no more than 2 prior lines of treatment. Participants were stratified in 4 different groups: adipocytic sarcomas, leiomyosarcomas, synovial sarcomas and others. While the adipocytic sarcoma cohort was prematurely closed due to lack of efficacy, pazopanib achieved positive results in the remaining groups. For instance, PFS rates at 12 weeks were 44% in the leiomyosarcoma cohort, 49% in synovial sarcoma and 39% in other sarcoma subtypes (94). Such encouraging data led to the conduction of a randomized, double-blind, placebo-controlled phase III trial in patients with metastatic non-adipocytic sarcomas, the PALETTE study. A total of 369 patients were treated within this trial with either pazopanib or placebo in a 2:1 randomization in favour of the antiangiogenic agent. Median PFS was superior with pazopanib compared to placebo (4.6 months vs 1.6 months), the p-value for the difference being <0.0001. OS was also higher in the pazopanib arm (12.5 months vs 10.7 months) although this difference was not statistically significant (15). Pazopanib is currently licensed for the treatment of non-adipocytic sarcomas thanks to these efficacy results. Furthermore, signs of activity have also been recently reported in rare sarcomas such as solitary fibrous tumor (95) or desmoid tumor/aggressive fibromatosis (96).

3.1.2.5 Thalidomide

Thalidomide is an old drug with antiangiogenic characteristics that were first described in the early nineties (97). Its activity in sarcomas has been assessed in several studies focused on gynecologic soft tissue sarcomas with unsuccessful results. The first report, published in 2006, was a phase II trial in which 17 patients affected by soft tissue sarcomas or carcinosarcomas of gynecological origin were treated with an escalating

regimen of thalidomide. The treatment was poorly tolerated and the survival analysis showed a median PFS of 1.84 months and a median OS of 6.64 months. Owing to the lack of activity and high toxicity observed, the study was closed prematurely (98). Two more trials failed to demonstrate efficacy of this compound in sarcomas. The first of them also focused on patients with gynecologic soft tissue sarcomas. Thirty patients with persistent or recurrent uterine leiomyosarcoma were enrolled and again thalidomide was found to be excessively toxic: only 2 patients were able to receive more than 4 cycles. Median PFS was 1.9 months and median OS was 8.3 months. Moreover, no responses were seen (99). The last trial published to date with thalidomide in sarcomas also showed discouraging results. The target population were patients with unresectable or metastatic leiomyosarcoma. In this study the treatment was a combination regimen with temozolomide and thalidomide. Only 2 out of 24 patients (10%) achieved PR and 5 (24%) had SD for at least 6 months. Although the authors concluded that the regimen showed signs of activity, they point out that the role of thalidomide in sarcomas is at best uncertain (100).

3.1.2.6 Taxanes (paclitaxel)

Paclitaxel is an antimicrotubule drug with antiangiogenic properties (101). Interestingly, it has shown activity in only one specific type of sarcoma: angiosarcoma. The first case series with successful results was reported in 1999. In this series, all 9 patients with scalp or face angiosarcoma responded after being treated with different schedules of paclitaxel with a median duration of response of 5 months (102). Similar encouraging results were observed in a retrospective study by European Organisation

for Research and Treatment of Cancer (EORTC). Nearly half of the 32 reported patients had received at least one line of previous treatment with doxorubicin. Paclitaxel also showed special activity in scalp angiosarcomas, with a RR of 75%. However, it was also effective in patients with other sites of disease (RR 58%) and the RR in the overall population was 62%. The benefit in time to progression was also higher in the scalp angiosarcoma cohort compared to other sites and to the whole group (9.5, 7 and 7.6 months, respectively) (103). A prospective phase II study conducted by the French Sarcoma Group with weekly paclitaxel in unresectable angiosarcomas showed less promising results. The RR in the 30 patients enrolled was not as high as in the previously reported case series but weekly paclitaxel showed interesting activity in angiosarcomas with PFS rates at 2 and 4 months of 74% and 45%, respectively. In addition, median time to progression was 4 months and median overall OS was 8 months. Interestingly, 2 out of 3 patients with breast angiosarcomas were found to have a pathological CR at surgery performed after achieving radiological PR. Moreover, the treatment showed a very favourable toxicity profile (104).

Results observed in these studies provided a rationale to consider paclitaxel as a valid alternative for patients affected by angiosarcoma. Furthermore, data from some series of cases suggest that its efficacy can be similar to other drugs. The first small retrospective study that indirectly compared paclitaxel to another treatment in this subset of patients was published in 2005. Data from 8 patients treated in first line with paclitaxel and 6 treated with pegylated liposomal doxorubicin were collected. Of the 8 patients that received paclitaxel, 3 achieved PR and 2 achieved CR as best response.

On the other hand, 3 out of 6 patients treated with pegylated liposomal doxorubicin had PR and 2 had SD (105). Another retrospective study compared the outcomes of 117 angiosarcoma patients treated with either single agent doxorubicin or weekly paclitaxel in the first line setting. From 34 patients evaluable for response in the doxorubicin group, 2 (6%) had CR, 8 (23.5%) had PR and 10 (29.5%) had SD. In the weekly paclitaxel cohort, 9 out of 68 patients evaluable for response (13%) achieved CR, 27 (40%) achieved PR and 20 (29.5%) achieved SD. Cutaneous angiosarcomas were again found to be more sensitive to paclitaxel whereas doxorubicin efficacy was not affected by tumor site (106). The last indirect comparison published to date assessed the impact of different modalities of treatment in patients affected by metastatic angiosarcoma: doxorubicin-based regimens, weekly paclitaxel, metastasectomy, other chemotherapy regimens or palliative care exclusively. The retrospective data extracted from 149 cases showed that doxorubicin, paclitaxel and metastasectomy improved survival compared to palliative care. Furthermore, the authors also concluded that doxorubicin-based regimens and weekly paclitaxel are similar in terms of efficacy (107).

3.2 The mTOR pathway

mTOR is a serine/threonine kinase that plays a central role in the phosphatidyl inositol 3'-kinase (PI3K)-AKT pathway, a cell signaling hub in which converge systems such as the insulin growth factor (IGF), the fibroblast growth factor (FGF) or the epidermal growth factor (EGF) pathways (108). It has been described 2 different mTOR complexes: mTOR complex 1 (mTORC1) and 2 (mTORC2). Both mTOR complexes share

the catalytic mTOR subunit, and also mammalian lethal with sec-13 protein 8 (mLST8, also known as GbL), DEP domain containing mTOR-interacting protein (DEPTOR), and the Tti1/Tel2 complex. In contrast, regulatory-associated protein of mammalian target of rapamycin (raptor) and proline-rich Akt substrate 40 kDa (PRAS40) are specific to mTORC1, whereas rapamycin-insensitive companion of mTOR (rictor), mammalian stress-activated map kinase-interacting protein 1 (mSin1) and protein observed with rictor 1 and 2 (protor1/2) are only part of mTORC2 (109, 110). mTORC1 is strongly inhibited by sirolimus (also known as rapamycin) while mTORC2 is not sensitive to this drug. Most of the work done so far in the study of the relationship between mTOR and cancer has been focused on mTORC1 due to its inhibition by sirolimus and its derivatives (111). However, dual PI3K/mTOR inhibitors and ATP-competitive mTORC1/mTORC2 inhibitors have been recently developed so a deeper knowledge of the pathway has become available (112).

Activation of the mTOR pathway by different environmental and nutritional stimuli triggers transduction of proliferative signals by the phosphorylation of two key downstream effectors, the p70 S6 kinase and the eukaryotic initiation factor 4E binding protein 1 (4EBP-1) (113). These proteins are involved in the biosynthesis of ribosomes and translation of mRNA necessary for normal cell-cycle regulation (114). The correlation between mTOR pathway abnormalities and carcinogenesis has been extensively reported (115). Indeed, up to half of all human tumors have been found to be somehow driven by alterations in the mTOR pathway (116). Amplification or activating mutations in genes encoding upstream tyrosine kinase receptors (117), activating mutations of *PI3KCA* (the gene encoding the PI3K catalytic subunit p110a) (118) or deletion/inactivation of tumor suppressors genes (119-121) activate the mTOR

pathway leading to uncontrolled tumor cell proliferation. mTOR deregulation is also involved in some familiar cancer syndromes. Thus, germline mutations in phosphatase and tensin homolog (PTEN) (the main negative regulator of PI3K/AKT/mTOR signaling) are found in more than 70% of patients with the Cowden syndrome, which is related with an increased risk for breast, endometrial, thyroid and renal carcinomas (122). The LKB1 tumor suppressor is lost in Peutz–Jeghers syndrome, which produces the development of intestinal polyps and increased risk of colorectal cancer (123). Neurofibromatosis type 1 (NF1) is caused by mutations in the *NF1* gene, whose product (termed neurofibromin) activates mTORC1 leading to the development of both benign and malignant tumors of the central and peripheral nervous system (124). In addition, the mTOR pathway is also critical in some tumor microenvironment processes such as angiogenesis since mTORC1 activates the transcription and translation of HIF-1α (125).

3.2.1 Rationale for the inhibition of the mTOR pathway in sarcomas

Upregulation of growth factors or mutations in tyrosine kinase receptors that belong to the mTOR network have been reported to be involved in the development of various sarcomas (126).

For instance, aberrations in some of the components of the IGF network triggers sarcomagenesis through mTOR signaling. The IGF system consists of 3 ligands (IGF-I, IGFII and insulin), 4 cell-membrane receptors (IGF-1R, insulin receptor isoform A (IR-A), hybrid receptors and IGF receptor type 2 (IGF-2R)) and 6 IGF binding proteins (IGFBP-1–6) (127). In humans IGF-II is the predominant circulating IGF, with plasma levels 3-7

fold higher than IGF-I (128). Some malignancies, especially sarcomas, secrete large amounts of incompletely processed forms of IGF-II which reflects in high circulating levels of this protein resulting in hypoglycaemia (129). Enhanced expression of IGF-II is present in some types of sarcoma such as embryonal rhabdomyosarcoma through at least 2 different mechanisms: loss of imprinting (LOI) process leading to elevated mRNA levels of the IGF-II gene (*IGF2*) and loss of heterozygosity (LOH) of *IGF2* which associates with duplication of the paternal allele of *IGF2* resulting in the expression of the 2 paternal genes. Moreover, enhanced loss of inhibitors of IGF-II expression such as p53 play a role in increased expression observed in several sarcomas (130). IGF-1R is a transmembrane tyrosine kinase receptor highly expressed in many human malignancies including sarcomas. Loss of transcriptional repressors such as p53 or increased expression of transcriptional activators such as chimeric transcription factors lead to aberrant expression of IGF-1R. This has been reported in alveolar rhabdomyosarcoma (131). IGF-2R, on the other hand, is involved in the regulation of the extracellular concentration of IGF-II (132). In many tumours, loss of IGF-2R expression or function has been observed by either LOH or inactivating mutations (133, 134). On the contrary, overexpression of IGF-2R leads to reduced tumour growth and delayed tumourigenesis in *in vivo* models, therefore suggesting that IGF-2R acts as a tumour suppressor (135, 136). But it is in several specific sarcoma subtypes where alterations in the IGF are especially relevant. Thus, overexpression of IGF-II and/or constitutive activation of insulin receptor substrate 1 (IRS-1) has been found in up to 50% of all leiomyosarcomas (137-139). Also, the axis IGF-1R/IGF-II plays a crucial role in the pathogenesis of synovial sarcomas. Indeed, *IGF2* is highly expressed when cells are transfected with the characteristic synovial sarcoma chromosomal translocation SS18-

SSX1 or SSX2 (140, 141). Moreover, high expression of IGF-1R in synovial sarcoma is associated with the development of metastases (142). In addition, IGF-1R amplifications correlated with poor survival have been described in malignant peripheral nerve sheath tumor (MPNST), a rare sarcoma (143). A number of phase II clinical trials with IGF pathway inhibitors have been conducted in recent years with not very encouraging results (144).

The FGF pathway is another key cellular network which activation eventually leads to mTOR-mediated cell proliferation. Several aberrations, including gene amplifications, point mutations or chromosomal translocations have been described across solid tumors (145) and its importance in different types of sarcomas has been recently described. FGF receptor (FGFR) substrate 2 (FRS2) (an adaptor protein that plays a critical role in FGFR signalling) is located on chromosome 12q13-15, a region that is frequently amplified in liposarcomas. Inhibition of FGFR in *in vitro* and *in vivo* models of liposarcoma represses cell proliferation and viability (146). Also, disruption of the FGF signaling pathway in synovial sarcoma by specific inhibitors of FGFR induces cell cycle arrest leading to significant growth inhibition both *in vitro and in vivo* (147).

The relevance of the EGF pathway in sarcomas has also been assessed. EGF receptor (EGFR) expression was analyzed in 281 tumor samples from patients affected by soft tissue sarcomas and it was reported positive in 168 of 281 (60%) cases. Moreover, EGFR overexpression was found to be a negative prognostic factor strongly associated with histologic grade (148). Treatment with EGFR inhibitors in MPNST cell lines, a subtype of sarcoma where overexpression of EGFR has been described, induces a

blockade in the mTOR pathway and subsequently reduces cell proliferation (149-151). Similar results have been observed in synovial sarcoma (151, 152).

Alterations in the main mTOR pathway controller, PTEN, have also been found to be directly implicated in the development of sarcomas. Thus, it has been reported that genetic inactivation of PTEN in murine models induces the formation of leiomyosarcomas, which is consistent with the constitutive activation of AKT and mTOR observed in up to 90% of cases of leiomyosarcoma. Inhibition of mTOR by the sirolimus derivative everolimus substantially decelerates tumor growth and improves mice survival (153). Also, it has been demonstrated that loss of function of PTEN activates the mTOR pathway favouring the development of MPNST (154) and mTOR inhibition by everolimus has antitumor activity on MPNST cell lines (155). Furthermore, aberrant AKT activation drives the formation of liposarcomas (156) whereas deletions in some other mTOR pathway tumor suppressors such as tuberous sclerosis complex 1 and 2 (*TSC1* and *TSC2*) and *NF1* are associated with both benign and malignant mesenchymal tumors (157, 158).

3.2.2 mTOR inhibitors in sarcomas: clinical experience

3.2.2.1 Sirolimus

Sirolimus was the first compound developed that was able to inhibit mTOR (159). Some its derivatives, namely everolimus, temsirolimus and ridaforolimus, have been successfully assessed in phase III trials in different malignancies (16, 160-163).

Sirolimus is a macrolide that prevents the phosphorylation of S6 and 4EBP-1 and therefore their activation (164). A study published in 2012 reported the results of 3 phase I trials conducted in advanced cancer patients using an adaptive escalation design to find the dose of oral, weekly sirolimus alone or in combination with either ketoconazole or grapefruit juice. Results showed that sirolimus can be feasibly administered orally, once weekly, achieving similar blood concentrations as its intravenous derivative temsirolimus. Among the 138 subjects enrolled, the most commonly observed toxicities were hyperglycemia, hyperlipidemia and lymphopenia (52%, 43%, and 41%, respectively). Interestingly, one PR was observed in a patient with an infrequent type of sarcoma, epithelioid hemangioendothelioma (165).

The activity of sirolimus has also been reported in a short case series of 3 patients with advanced perivascular epithelioid cell tumors (PEComas), a family of mesenchymal neoplasms that depend on aberrant mTOR signaling and for which there is no effective treatment. All patients achieved radiographic responses. Moreover, mTORC1 was found to be pathologically activated by loss of the TSC1/TSC2 tumor suppressor complex in the 3 cases (166).

Finally, in a rare gynecological sarcoma (endometrial stromal sarcoma), our group reported the case of a patient in which sirolimus likely contributed to the reversion of hormone manipulation resistance. PR was achieved following the addition of sirolimus to the hormone treatment and response was maintained for more than 2 years with minimal toxicity (167).

3.2.2.2 Temsirolimus

The activity of temsirolimus, a sirolimus derivative administered intravenously, was assessed in a phase II study in patients with advanced soft tissue sarcoma. A total of 41 chemotherapy-naïve patients were enrolled with disappointing results: only 2 patients (1 undifferentiated fibrosarcoma and 1 uterine leiomyosarcoma) achieved a confirmed PR lasting 3 and 17 months, respectively. The median OS was 7.6 months and, at the time of the report, 39 patients (95%) had progressed with a median time to progression of 2.0 months. Regarding toxicity, 43% of patients experienced at least grade 3 adverse events related to treatment. Such results led the authors to conclude that temsirolimus had limited clinical activity and moderate toxicity in the population of the study (168).

3.2.2.3 Ridaforolimus

Ridaforolimus is another sirolimus-derived compound formerly known as deforolimus. A multicenter, open-label, single-arm, phase II trial was conducted to assess the antitumor activity of ridaforolimus in patients with distinct subtypes of advanced sarcomas. A total of 212 patients with metastatic or unresectable soft tissue or bone sarcomas were treated in four separate histologic cohorts: bone sarcoma, leiomyosarcoma, liposarcoma and other sarcomas. A total of 61 heavily pretreated patients (28.8%) achieved clinical benefit (defined as CR or PR or SD ≥ 16 weeks), with a confirmed RR of 1.9% (4 patients has PR: 2 with osteosarcoma, 1 with spindle cell sarcoma and 1 with malignant fibrous histiocytoma). Median PFS was 15.3 weeks whereas median OS was 40 weeks. Treatment toxicity was generally mild or moderate

and consisted primarily of stomatitis, mucosal inflammation, mouth ulceration, rash and fatigue. Overall, PFS results compared favorably with historical metrics (169).

Subsequently, a phase III trial was conducted. It was a double-blind, placebo-controlled phase III study which randomized sarcoma patients who had achieved CR, PR or SD after 1, 2 or 3 lines of chemotherapy to receive placebo or ridaforolimus as maintenance treatment. A total of 702 patients received blinded study drug. Ridaforolimus showed signs of activity, inducing a mean 1.3% decrease in target lesion size versus a 10.3% increase with placebo. In addition, it achieved a modest but statistically significant improvement in PFS compared with placebo: median PFS was 17.7 weeks versus 14.6 weeks per independent review in favour of the mTOR inhibitor. The difference in OS (90.6 weeks versus 85.3 weeks for ridaforolimus and placebo, respectively) was not statistically significant. As expected, toxicity was more common in the ridaforolimus group of patients. The most frequent adverse events observed included stomatitis 77.8%, infections 51.6%, fatigue 35.6% and thrombocytopenia 33.5%. Overall, the percentage of discontinuation rate due to adverse events in the ridaforolimus cohort was 14.6% (16). These positive phase III results, although with a limited clinical applicability, further support the rationale for inhibiting the mTOR pathway in sarcomas.

4. Rationale for combinatory regimes of cytotoxic agents and targeted therapy

4.1 Combination of chemotherapy plus antiangiogenic agents

As discussed above, antiangiogenic agents have achieved successful results as single agents in different tumor types in recent years (170). However, they are not a curative treatment in cancer and their efficacy is limited by mechanisms of resistance (171). Several strategies for improving their efficacy have been studied, one of them being the combination with cytotoxic agents (172). This approach has achieved positive results, for instance, with combinations of bevacizumab with different chemotherapy agents. Thus, bevacizumab plus chemotherapy significantly prolongs OS in patients with metastatic colorectal cancer (CRC) (173), recurrent/advanced non-small cell lung cancer (NSCLC) (174) or extends PFS in patients with advanced ovarian cancer (175, 176).

The mechanisms by which antiangiogenic agents increase the efficacy of chemotherapy are still not fully understood but it seems clear that the combination of both agents deprives the tumors of nutrients and kills highly proliferative tumor cells (177). The tumor microenvironment has a structurally and functionally abnormal vasculature, with increased vessel permeability, dilatation and tortuosity, reduced pericyte coverage, and abnormal basement membranes. This is mainly because of an imbalance between pro- and anti-angiogenic factors (178, 179). As a consequence, tumor blood flow is impaired and this, together with compression of the blood vessel

by the growing cancer, results in high interstitial fluid pressure, hypoxic regions within the tumor, and ultimately reduced drug delivery (180, 181). Antiangiogenesis agents are able to modify the tumor microenvironment since abnormal microvessels are destroyed and the remaining vessels are remodelled (182). These changes, termed as 'vascular normalization' lead to a transient increase in vascular patency, a drop interstitial fluid pressure and alleviation of hypoxia, providing a window of opportunity for the delivery of drugs, thus achieving a better therapeutic outcome (183, 184). Whether these morphological changes are accompanied by functional modifications, such as improved drug delivery, is still unknown (71, 185). In the clinical setting, careful dosing, scheduling and sequencing of treatments is essential to optimize the efficacy of combinations due to the potential risk for overlapping toxicities (186, 187). Well-designed phase I trials with a solid preclinical rationale are therefore needed (188).

4.2 Combination of chemotherapy plus mTOR inhibitors

Activation of the PI3K/AKT/mTOR pathway is a potential mechanism of resistance to conventional chemotherapy in carcinomas (189). Therefore, combining chemotherapeutic agents with inhibitors of the PI3K/AKT/mTOR pathway such as mTOR inhibitors is a sensible therapeutic approach to be assessed in sarcomas (190).

Combinations of sirolimus and some of its derivatives with chemotherapy have been assessed in preclinical models of different tumor types. For instance, treatment of neuroblastoma mice models with sirolimus and the vinca alkaloid vinblastine (a chemotherapeutic drug that blocks mitosis by inhibiting the assembly of microtubules)

results in inhibition of tumor growth and angiogenesis, with an increase in survival compared to either compound alone (191, 192). In a panel of pediatric tumors, sirolimus was assessed in combination with different chemotherapeutic agents (melphalan, cisplatin, cyclophosphamide and vincristine). Results *in vitro* showed subadditive or additive effects whereas therapeutic enhancement was found for sirolimus plus cyclophosphamide and vincristine *in vivo*. These combinations were significantly more effective than each agent alone (193). In hepatocellular carcinoma, low doses of vinblastine plus sirolimus induces potent antiangiogenic effects (194).

In vitro, everolimus has demonstrated synergistic effects when combined with rituximab, doxorubicin and vincristine mostly through the induction of cell cycle arrest in diffuse B cell large lymphoma (195) and in mantle lymphoma (196). Moreover, everolimus has been found to dramatically enhance cisplatin-induced apoptosis in wild-type p53 (but not in mutant p53 tumor cells) by inhibiting p53-induced p21 expression. This provides a strong rationale for combining DNA damaging agents with everolimus (197).

On the other hand, an additive cytotoxicity in malignant glioma cell lines and an enhancement in tumor growth inhibition in xenograft models have been found when temsirolimus is administered together with cisplatin and with the topoisomerase I inhibitor camptothecin (198). Temsirolimus also showed significant antitumor activity in pancreatic cancer xenograft models as both single agent and in combination with gemcitabine, with improved mice survival with the combination compared to each

drug alone (199). In melanoma models, a synergistic antitumor effect was observed *in vitro* with the treatment regimen of temsirolimus plus cisplatin (200), as well as an increase in the chemotherapeutic efficacy with the combination of temsirolimus and dacarbazine (201). In a different malignancy, small cell lung cancer (SCLC), temsirolimus has been found to be able to restore cisplatin sensitivity in cell lines selected for cisplatin resistance as well as in cell lines derived from patients who failed to cisplatin treatment (202).

Finally, ridaforolimus in combination with doxorubicin and with carboplatin plus paclitaxel has been assessed preclinically in sarcomas and in endometrial carcinoma, respectively. In sarcomas, the combination of ridaforolimus and doxorubicin was found to be either additive or moderately synergistic in all 6 cell lines evaluated. Similarly, the effects of the ridaforolimus and carboplatin plus paclitaxel combination in endometrial lines were generally additive. *In vivo*, potent antitumor activity of ridaforolimus associated with inhibition of mTOR signaling was observed in both sarcoma and endometrial xenograft models (203).

Taken together, these data indicate that combining mTOR inhibition with chemotherapy is a potentially effective approach in sarcomas treatment.

5. Hypothesis and objectives

5.1 Hypothesis

Inhibition of angiogenesis and the mTOR pathway in sarcomas in combination with active cytotoxic agents enhances the anti-tumor activity of each of both strategies alone without significant toxicity.

5.2 Primary objectives

- To assess the safety and to determine the recommended dose (RD) for the clinical development in sarcomas of two combinations of cytotoxic agents and targeted therapies: ifosfamide plus sorafenib and gemcitabine plus sirolimus.

- To preliminary assess signs of clinical activity of the combinations of ifosfamide plus sorafenib and gemcitabine plus sirolimus in advanced sarcomas and other solid tumors.

5.3 Secondary objectives

- To assess the pharmacokinetic (PK) properties the combinations of ifosfamide plus sorafenib and gemcitabine plus sirolimus.

- To assess the pharmacodynamic properties of the combination of gemcitabine plus sirolimus.

- To assess the anti-tumor activity of the combination of ifosfamide plus sorafenib in *in vitro* models of sarcoma.

- To assess the anti-tumor activity and the effects on the mTOR pathway of the combination of gemcitabine plus sirolimus in *in vitro* and *in vivo* models of sarcoma.

6. Articles

6.1 Phase I trial of sorafenib in combination with ifosfamide in patients with advanced sarcoma: a Spanish group for research on sarcomas (GEIS) study

Invest New Drugs
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PHASE I STUDIES

Phase I trial of sorafenib in combination with ifosfamide in patients with advanced sarcoma: a Spanish group for research on sarcomas (GEIS) study

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Summary *Background* This phase I trial assessed safety, pharmacokinetics (PK), dose limiting toxicity (DLT), maximum tolerated dose and recommended dose (RD) of the combination of sorafenib plus ifosfamide in patients with advanced sarcoma. *Methods* Twelve sarcoma patients (9 soft-tissue, 3 bone sarcoma) were treated with sorafenib plus ifosfamide (starting doses 200 mg bid and 6 g/m^2 respectively). A 3+3 dose escalation design with cohorts of 3–6 patients was used. A study to assess the in vitro efficacy of the combination was also conducted. *Results* Three DLTs were observed: fatigue grade 4 with sorafenib 400 mg bid plus ifosfamide 6 g/m^2 and encephalopathy and emesis grade 3 with sorafenib 400 mg bid plus ifosfamide 7.5 g/m^2. Other toxicities included diarrhea, hand-foot syndrome, mucositis, neutropenia, skin rash and thrombocytopenia. There were no relevant effects on PK of sorafenib but an increase in ifosfamide active metabolite 4-hydroxy-ifosfamide was observed. Eight patients achieved stable disease lasting more than 12 weeks. An additive effect was observed in vitro. *Conclusions* RD was sorafenib 400 mg bid plus ifosfamide 6 g/m^2, allowing administration of active doses of both agents. Limited preliminary antitumor activity was also observed. A phase II study is currently ongoing.

Keywords GEIS · Ifosfamide · Phase I · Sarcoma · Sorafenib

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Introduction

Soft tissue sarcomas (STS) are an uncommon heterogeneous group of malignant tumors of mesenchymal origin very often associated with bad prognosis. For most advanced STS types, chemotherapy is currently the only available treatment. Unfortunately, a very limited number of useful drugs are active against these diseases. Doxorubicin is widely considered the standard first-line treatment although its response rates of 10–30 % are still very poor [1–4]. Ifosfamide has also a well-established activity [5, 6] and is often administered either associated with anthracyclines or alone as a second-line chemotherapy treatment. Other drugs such as DTIC, gemcitabine and temozolomide have shown modest activity as second-line agents [7, 8]. Thus, it is necessary to identify new active agents to improve therapy for patients with advanced STS.

In some studies, most STS showed VEGF expression and elevated serum VEGF levels are found to correlate with higher histologic tumor grade [9, 10]. Additionally, inhibition of VEGFR is associated with anti-tumor activity in preclinical

models of sarcoma [11, 12]. For these reasons, inhibition of VEGFR seems to be a reasonable approach to explore in the treatment of STS. Sorafenib (BAY 43–9006) is an orally available, small molecule multi-kinase inhibitor of VEGFR, PDGFR and RAF with demonstrated activity in the treatment of renal cell cancer [13] as well as in other malignancies. Thus, effects of sorafenib in certain sarcoma cell lines have been studied showing signs of activity [14–18]. Furthermore, a phase II trial published in 2009 showed encouraging results, achieving progression free survival (PFS) rates for leiomyosarcoma and angiosarcoma patients comparable with standard cytotoxic agents [19]. Some other studies have also been conducted to explore the efficacy of sorafenib alone in small cohorts of sarcoma patients with promising results [20, 21].

Preclinical studies also suggest that the inhibition of VEGF pathway with drugs like sorafenib together with cytotoxic agents result in additive anti-tumor activity [22], justifying combination studies. A recent trial, however, reported an unexpectedly high incidence of cardiac toxicity in patients with STS treated with bevacizumab (a monoclonal antibody that binds VEGF) in combination with doxorubicin [23]. This finding suggests that a potential increase in doxorubicin cardiotoxicity when inhibiting the VEGF pathway cannot be ruled out. The association of sorafenib with ifosfamide, the other established active agent against STS, may improve the efficacy of single-agent ifosfamide minimizing the risk of cardiac toxicity. Thus, this phase I trial was designed to establish the recommended dose (RD) and to assess the safety of the combination of sorafenib and ifosfamide. A preclinical study to evaluate the in vitro activity of this treatment was also conducted.

Materials and methods

Patients

To be enrolled in this study, patients had to meet the following eligibility criteria: diagnosis of advanced soft tissue or bone sarcoma previously treated with anthracyclines (or ineligibles for that treatment), no prior treatment with ifosfamide, age ≥18 and <72 years, Eastern Cooperative Oncology Group performance status (ECOG PS) 0–1 and either measurable or evaluable disease. Adequate bone marrow, renal and hepatic function were mandatory and were defined as: absolute neutrophil count≥1.5×10^9/L, platelets≥100×10^9/L, hemoglobine≥ 9 g/dl, bilirubin≤1.5×upper limit of normal (ULN), aspartate transaminase and alanine transaminase≤2.5×ULN, creatinine≤1.5×ULN, creatinine clearance≥50 mL/min and INR≤1.5. Patients with uncontrolled hypertension, history of coagulopathy, thrombotic or embolic events were considered ineligibles. Other exclusion criteria were treatment with chemotherapy or radiotherapy within 3 weeks prior, pregnancy, life expectancy<12 weeks, other tumors treated with curative intention in the last 3 years and known central nervous system metastasis.

All patients signed written informed consent and the study was conducted according to local and national ethical review board approval, the Declaration of Helsinki and standards of Good Clinical Practice.

Study design and drug dosage, escalation and administration

Trial was performed in 4 specialized Spanish centers belonging to the Spanish Group for Research on Sarcomas (GEIS) using a standard 3+3 dose escalation phase I design with cohorts of 3–6 patients. If less than one-third of patients at a dose level experienced a dose limiting toxicity (DLT), dose escalation continued. If more than one-third but less than two-thirds of patients at a dose level had a DLT, 3 additional patients were enrolled at that same dose level. If two-thirds or more of patients at a dose level experienced a DLT, the dose was considered toxic and the next cohort of patients was included at the next lower dose level. Dose escalation within a patient was not permitted.

Dose levels are described in Table 1. Patients remained on study treatment for as long as the investigator considered it has some benefit for the patient and there was no evidence of progressive disease (PD) or unacceptable toxicity. A maximum of 6 cycles of treatment per patient were allowed. However, treatment with single-agent sorafenib beyond 6 cycles was allowed at investigator's criteria if there was no evidence of PD.

Routine clinical and laboratory assessments were conducted on a weekly basis. Toxicity was graded using the National

Table 1 Dose levels

Dose levels
Level 1
- Sorafenib 200 mg bid po (started on day 2)
- Ifosfamide 2.0 g/m^2 iv over 4 h×3 consecutive days
- Mesna 400 mg/m^2 iv at 0, 4 and 8 h after ifosfamide
Level 2
- Sorafenib 400 mg bid po (started on day 2)
- Ifosfamide 2.0 g/m^2 iv over 4 h×3 consecutive days
- Mesna 400 mg/m^2 iv at 0, 4 and 8 h after ifosfamide
Level 3
- Sorafenib 400 mg bid po (started on day 2)
- Ifosfamide 2.5 g/m^2 iv over 4 h×3 consecutive days
- Mesna 500 mg/m^2 iv at 0, 4 and 8 h after ifosfamide
Level 4
- Sorafenib 400 mg bid po (started on day 2)
- Ifosfamide 3.0 g/m^2 iv over 4 h×3 consecutive days
- Mesna 600 mg/m^2 iv at 0, 4 and 8 h after ifosfamide

Cancer Institute Common Toxicity Criteria version 3.0 (NCI-CTCAE v3.0). DLT was defined as any of the following: absolute neutrophil count$<0.5\times10^9$/L over≥5 days or associated with fever≥38.5 °C, platelets $<50\times10^9$/L or any grade 3–4 non hematological toxicity (excluding nausea and vomiting non refractory to antiemetic treatment) during cycle 1 of treatment. Maximum tolerated dose (MTD) was defined as the highest dose level in which 2 or more patients experienced DLT. Next lower dose level was considered as RD.

In order to assess tumor response to treatment, thorax-abdomen-pelvis CT scan was performed every 6 weeks. Response Evaluation Criteria in Solid Tumors version 1.0 (RECIST v1.0) were used.

Pharmacokinetics

A total number of 32 blood samples from each patient were obtained for pharmacokinetic (PK) analysis. Blood samples (6 ml) for the determination of PK of ifosfamide and its metabolite 4-hydroxy-ifosfamide were collected on day 1 of the first cycle before the start of the ifosfamide infusion and at 1, 2, 4 (end of infusion), 4.5, 5, 6, 7, 8, 10, 12 and 24 h thereafter. The 24-h sample was collected prior to start of the first dose of sorafenib on day 2. On day 1 of cycle 2 the collection of blood samples was repeated according to the same time schedule as on day 1 of cycle 1, while the dosing of sorafenib was continued. In addition, blood samples were collected prior to the morning dose of sorafenib and at 0.5, 1, 2, 4, 8, 10 and 12 h thereafter in order to determine the PK parameters of sorafenib.

Plasma samples for the determination of ifosfamide and 4-hydroxy-ifosfamide were preserved with 2 M semicarbazide hydrochloride solution pH 7.4 and then stored at −70 °C. Plasma samples for the determination of sorafenib were stored at −20 °C. Plasma concentrations of ifosfamide, 4-hydroxy-ifosfamide and sorafenib were analyzed using fully validated LC-MS/MS assay methods. Quality control (QC) samples for all three analyses were determined with an accuracy ranging from 96.3 % to 105.2 % (ifosfamide), from 94.4 % to 103.7 % (4-hydroxy-ifosfamide), and from 102.7 % to 108.3 % (sorafenib), respectively. The precision was better than 7.6 % for all three analyses.

The maximum plasma concentration (C_{max}) and area under the plasma concentration time curve from dosing to the last quantifiable concentration above the lower limit of quantification (AUC(0-tn)) were calculated using the model-independent (compartment-free) method and the PC program WinNonlin (Version 4.1; Pharsight Corporation).

In vitro study

Two cell lines acquired from Cell Lines Service (CLS) were used to assess the in vitro efficacy of the combination: SKLMS-1 and SW982 (leiomyosarcoma and synovial sarcoma respectively). Both cell lines were cultured in RPMI 1640 (Invitrogen) supplemented with 10 % heat-inactivated fetal bovine serum (FBS, Invitrogen) and were incubated at 37 °C in a humidified atmosphere of 5 % CO2 in air. Sorafenib (BAY 549085) was supplied by Bayer Corporation (West Haven, CT), and was dissolved in 100 % DMSO (Sigma). Ifosfamide (Sigma) was dissolved in 100 % sterile water. Sorafenib and ifosfamide were diluted with cell medium at 10 μM and 1 mM concentration respectively and then cells were treated with both drugs separately and in combination for 48 h. DMSO was added to cultures as control. Cell proliferation and cell death were determined by the Trypan Blue exclusion assay.

Results

Patient characteristics

From October 2007 to February 2009, a total of 12 patients were enrolled in this study. Demographics and baseline characteristics are shown in Table 2. Two additional patients were non evaluable because they were erroneously treated at a lower dose level (dose level 2 instead of dose level 3). Eleven out of 12 patients, (91.7 %) had received prior chemotherapy (at least 1 anthracycline based treatment in all cases). There was 1 patient ineligible for anthracyclines due to previous history of cardiopathy. The median number of previous chemotherapy lines was 1 (range 1–4) and the median number of previous agents was 1 (range 1–3). Eight patients (66.7 %) had radiation therapy before enrollment in the study.

Toxicity

Twelve patients were evaluable for safety. In the first cycle of therapy, DLT was observed in 3 patients. One out of 6 patients at dose level 2 experienced fatigue grade 4 and 2 out of 3 patients at dose level 3 experienced encephalopathy grade 3 and vomiting grade 3 respectively. Therefore, dose level 2 was defined as RD (Table 3). The 2 patients erroneously treated at a lower dose level received the dose level 2. One of them experienced a DLT, febrile neutropenia, in the first cycle, while the other one did not experience severe toxicity. However, the toxicity observed in these 2 patients does not change the definition of dose level 2 as RD, since DLTs were observed in a total of 2 out of 8 patients.

A total of 49 cycles were administrated, 15 at first level, 20 at second level and 14 at third level. Three patients (25 %) were treated at dose level 1, 6 patients (50 %) at dose level 2 and the remaining 3 patients received treatment at dose level 3. No patient was treated at dose level 4. The median of cycles of treatment administered per patient was 4.5 (range 1–6). Three

Table 2 Demographics and baseline characteristics

	Total patients (n=12)
Sex	
Male	6
Female	6
Age	
Median	53
Range	23–70
ECOG PS	
0	3
1	8
No data	1
Histology	
Liposarcoma	3
Leiomyosarcoma	2
Chondrosarcoma	2
Angiosarcoma	1
Hemangioperycitoma	1
Chordoma	1
Synovial sarcoma	1
Undifferentiated sarcoma	1
Primary tumor site	
Lower limb	4
Retroperitoneum	2
Head/neck	1
Chest wall	1
Other	4
Previous treatment	
Surgery	11
Chemotherapy	11
Radiotherapy	8

patients (25 %, 1 at each dose level) experienced a delay in the administration of ifosfamide. Two out of these 3 delays were due to hematological toxicity. Two patients had to reduce dose of sorafenib (1 due to hematological toxicity and 1 due to non-hematological toxicity) and none of the dose reductions happened at dose level 3 (1 at dose level 1 and 1 at dose level 2). Different circumstances lead to end of treatment: 3 patients (25 %) received the maximum number of cycles allowed (a total of 6), 4 patients (33.3 %) presented PD, 3 patients (25 %) experienced an adverse event (AE) grade 3–4 during first cycle of treatment, 1 patient (8.3 %) stopped treatment because of his own decision and there was 1 death (8.3 %) due to cancer. Toxicity is summarized in Table 4.

Table 3 DLTs at each dose level during first cycle

Dose level	Sorafenib (mg/12 h)	Ifosfamide (g/m^2)	Patients	Toxicity
1	200	6	0/3	
2	400	6	1/6	Asthenia G4
3	400	7.5	2/3	Encephalopathy G3 Emesis G3

Anti-tumor activity

Eleven out of 12 patients were evaluable for response. Eight patients (72.7 %) achieved stable disease (SD) lasting more than 12 weeks (3 patients at dose level 1, 3 patients at dose level 2 and 2 patients at dose level 3). The remaining 3 patients (27.2 %) experienced PD. No partial responses (PR) were observed.

Median PFS was 4.2 months (95 % CI, 1.1–5.8). PFR at 3 and 6 months were 64 % (95 % CI, 30.8–89.1) and 18 % (95 % CI, 2.3–51.8) respectively. Median overall survival (OS) reached 9.9 months (95 % CI, 5.1–NA) with a follow up of 48 months.

Pharmacokinetics

Sorafenib concentrations were within the range expected after continuous treatment at doses of 200 mg and 400 mg bid. Thus, no effects of concomitant ifosfamide on sorafenib PK were observed. Figure 1 shows the mean plasma concentration time courses of both ifosfamide and 4-hydroxy-ifosfamide following the iv infusion of 2 g/m^2 ifosfamide on day 1 of cycle 1 (without concomitant sorafenib) and on day 1 of cycle 2 (after multiple dosing with 400 mg sorafenib bid). Geometric mean C_{max} values (and geometric SD) for ifosfamide were 68.2 (1.16) mg/L on day 1 of cycle 1 and 69.1 (1.15) mg/L on day 1 of cycle 2. The corresponding geometric mean values for AUC(0–tn) were 722 (1.20) mg*h/L and 668 (1.09) mg*h/L, respectively, indicating no relevant change of these parameters upon concomitant dosing of 400 mg sorafenib bid. Mean C_{max} 4-hydroxy-ifosfamide values were 0.260 (1.16) mg/L (cycle 1) and 0.641 (1.05) mg/L (cycle 2) and mean AUC(0-tn) values were 1.71 (1.36) mg*h/L (cycle 1) and 3.86 (1.10) mg*h/L (cycle 2), respectively. These results seem to indicate an apparent increase in in both C_{max} and AUC(0-tn) of 4-hydroxy-ifosfamide by concomitant treatment with sorafenib. However, these results should be interpreted with caution, because the plasma concentrations of 4-hydroxy-ifosfamide were close to the lower limit of quantification (LLOQ) of the assay. As a consequence, the plasma concentration time course of this metabolite could be quantified for only 3.5–8 h. Due to these low concentrations, the mean PK parameters could only be compared on basis of 3 subjects.

Table 4 Toxicity

Toxicity	Dose level 1 (n=3)		Dose level 2 (n=6)		Dose level 3 (n=3)	
	All grades (n)	Grades 3–4 (n)	All grades (n)	Grades 3–4 (n)	All grades (n)	Grades 3–4 (n)
Alopecia	2	0	1	0	0	0
ALT increase	0	0	1	1	0	0
Amilase increase	0	0	1	1	0	0
Anemia	0	0	2	2	0	0
Anorexia	2	0	2	0	1	0
AST increase	0	0	1	0	0	0
Diarrhea	2	0	2	0	2	0
Encephalopathy	1	1	0	0	1	1
Fatigue	3	0	4	2	2	0
Febrile neutropenia	0	0	2	2	0	0
Hand-foot syndrome	0	0	1	0	1	0
Leucopenia	0	0	2	2	1	0
Mucositis	2	0	2	0	0	0
Myalgia	0	0	1	0	0	0
Nausea/Vomiting	2	0	3	0	2	1
Neutropenia	2	2	3	2	1	1
Skin rash	2	0	2	0	1	0
Thrombocytopenia	1	1	1	1	0	0

Toxicities reported at any time from first treatment administration to 30 days of last treatment administration are included

In vitro study

SKLMS-1 and SW982 cell lines were treated as described in materials and methods. Cell proliferation and death determined after 48 h of treatment showed a higher activity of the combination on both cell lines compared to each drug alone. An additive effect of the treatment was clearly demonstrated (Fig. 2). Moreover, the calculation of the Combinatory Index was 0.96, which confirms the superiority of the combination. Furthermore, phenotypic changes were more evident in cells when treated with both drugs, suggesting again more effectiveness with sorafenib and ifosfamide than with either of the drugs alone.

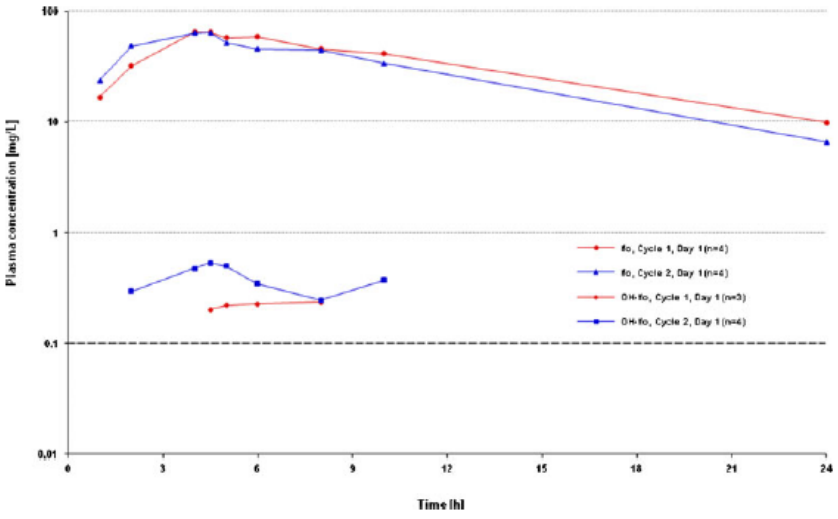

Fig. 1 Plasma concentration time courses of ifosfamide and 4-hydroxy-ifosfamide following the first infusions of 2 g/m^2 ifosfamide in cycle 1 and cycle 2 with and without concomitant administration of 400 mg sorafenib bid [geometric means]

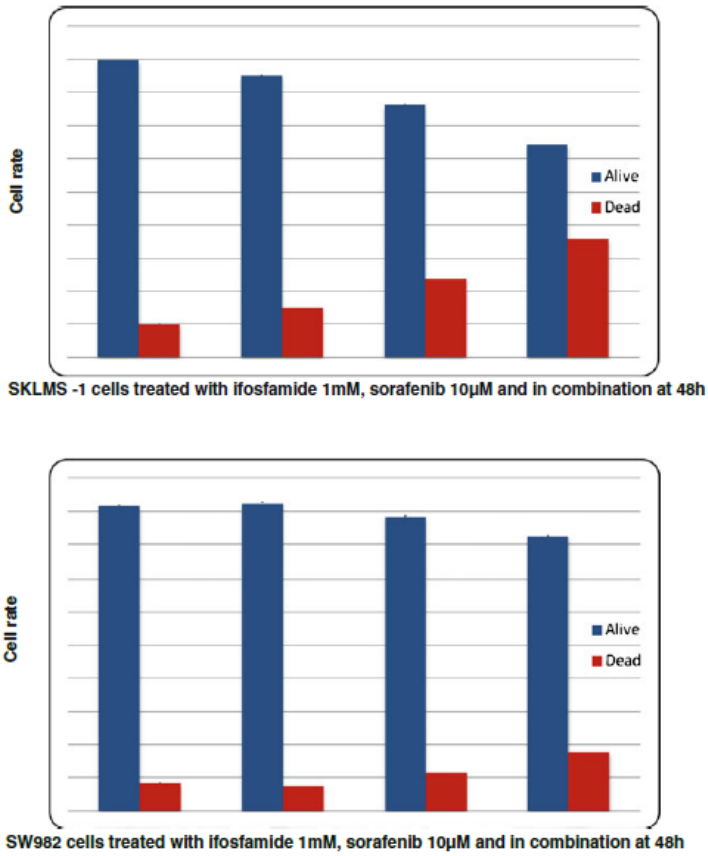

Fig. 2 Proliferation and cell death in vitro

Discussion

Our study demonstrates that this 2-drug regimen of sorafenib and ifosfamide is feasible and safe, allowing administration of active doses of both agents in patients with advanced sarcoma. The safety profile is very similar to each drug alone, showing no synergistic effect in toxicity. The most common adverse event registered was fatigue. This symptom was considered DLT in 1 patient treated at dose level 2 but it was not clinically significant in the majority of the cases. Other non-hematological toxicities such as stomatitis, diarrhea, skin rash or hand-foot syndrome were as frequent as expected with sorafenib or ifosfamide alone and were generally well tolerated and easily manageable. None of them reached grade 3–4. Vomiting and encephalopathy were the other 2 DLTs found, both relatively common and expected in patients treated with ifosfamide. Regarding hematological toxicity, mild granulocytopenia, thrombocytopenia and anemia were observed in most patients but modifications in the treatment schedule or dose were not necessary in almost every case. No cardiac or unexpected toxicity appeared with the combination and there was no toxic death. Despite the DLT observed in 1 non-evaluable extra patient, the treatment at RD proved to be safe. Therefore, the favorable toxicity profile leads us to recommend dose level 2 (sorafenib 400 mg bid and ifosfamide 6 g/m^2) as the optimal dose for further studies.

Phase II trials published with VEGFR inhibitors in sarcomas (sorafenib, sunitinib and pazopanib) achieved encouraging PFS data and some isolated responses that suggest promising activity of this new therapeutic approach [19, 24, 25]. Moreover, the recently reported results of the only randomized phase III trial to date with a VEGFR inhibitor (pazopanib) in patients with advanced soft tissue sarcoma showed a significant increase in PFS [26]. Thus, combining VEGFR inhibitors with cytotoxic drugs seems a reasonable strategy to be explored. In fact, our in vitro results reported here show higher efficacy when combining sorafenib and ifosfamide than with one of the drugs alone. But the experience previously published with treatment regimens combining cytotoxic agents with VEGFR inhibitors has shown some discouraging results not only in terms of safety but in PK profile as well. Thus, in a phase I trial that assessed toxicity and PK of the combination

of sunitinib and ifosfamide, it was found that ifosfamide produced decreased sunitinib blood levels because of CYP3A induction. In addition, synergy in toxicity with very high neutropenia rates was also observed and the final tolerable dose of sunitinib seems too low to be considered clinically relevant [27]. However, our study demonstrates that the safety profile of sorafenib allows its combination with ifosfamide at active doses of both drugs, making sorafenib a good candidate for combination regimes.

PK analysis showed no decrease in sorafenib and ifosfamide plasma levels with concomitant administration. Furthermore, 4-hydroxy-ifosfamide levels may rise with concomitant sorafenib with no additional effects on toxicity, indicating that sorafenib could enhance ifosfamide effect. Although these results should be considered with caution, as they are based on only a limited number of plasma samples and may be influenced by a high variability, they make this combination even more worth exploring. Indeed, with the limitations of a phase I study not designed to assess efficacy, it is worth noting the promising PFS results achieved according to the proposed EORTC efficacy criteria for treatment of sarcomas [28]. Nevertheless, the heterogeneous cohort of patients enrolled and the own design of the study make the interpretation of these efficacy results very limited. These clinical data, together with the additive effect of the combination observed in the in vitro associated study, creates interest in continuing investigation of this promising combination.

In conclusion, the lack of really effective treatments in mesenchymal tumors makes it necessary to find new therapeutic approaches to improve patients' outcome and angiogenesis inhibition seems worth exploring. Our phase I trial of the combination of sorafenib and ifosfamide focused on sarcomas showed a favorable PK profile and was demonstrated to be safe and feasible. Further studies to assess the activity of the combination of sorafenib and ifosfamide are warranted and a phase II trial is ongoing.

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6.2 Phase I study and preclinical efficacy evaluation of the mTOR inhibitor sirolimus plus gemcitabine in patients with advanced solid tumours

FULL PAPER

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Keywords: gemcitabine; sirolimus; rapamycin; mTOR; phase I

Phase I study and preclinical efficacy evaluation of the mTOR inhibitor sirolimus plus gemcitabine in patients with advanced solid tumours

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Background: We conducted a phase I study in patients with advanced solid tumours to identify the recommended dose, assess pharmacokinetics (PK), pharmacodynamic activity and preclinical antitumour efficacy of the combination of sirolimus and gemcitabine.

Methods: Nineteen patients were treated with sirolimus 2 or 5 mg daily and gemcitabine 800 or 1000 mg m^{-2} on days 1 and 8. Dose escalation depended on dose-limiting toxicity (DLT) rate during the first 3-week period. Paired skin biopsies were evaluated for phosphorylated S6 (pS6) as marker of mTOR (mammalian target of rapamycin) inhibition. Pharmacokinetics and preclinical evaluation of efficacy using two different sarcoma cell lines and leiomyosarcoma xenografts were also conducted.

Results: Three DLTs were observed: grade 3 transaminitis, grade 3 thrombocytopenia and grade 4 thrombocytopenia. Common treatment-related adverse events included anaemia, neutropenia, thrombocytopenia and transaminitis. Pharmacodynamic analyses demonstrated mTOR inhibition with sirolimus 5 mg and PK showed no influence of sirolimus concentrations on gemcitabine clearance. *In vitro* and *in vivo* studies suggested mTOR pathway hyperactivation by gemcitabine that was reversed by sirolimus. Tumour growth in leiomyosarcoma xenografts was dramatically inhibited by the treatment.

Conclusions: Recommended dose was sirolimus 5 mg per 24 h plus gemcitabine 800 mg m^{-2}. Antitumour activity in preclinical sarcoma models and mTOR signalling inhibition were observed. A phase II study is currently ongoing.

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that plays a central role in the phosphatidyl inositol 3′-kinase (PI3K)-AKT signalling pathway (Aoki *et al*, 2001; Sabatini, 2006). Activation of mTOR by different environmental and nutritional stimuli triggers transduction of proliferative signals by the phosphorylation of two key downstream effectors, the p70 S6

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kinase and the eukaryotic initiation factor 4E binding protein 1 (4EBP-1; Janus *et al*, 2005). These proteins are involved in the biosynthesis of ribosomes and translation of mRNA necessary for normal cell-cycle regulation (Mamane *et al*, 2006). The correlation between mTOR pathway abnormalities and carcinogenesis has been extensively reported (Shaw and Cantley, 2006; Hernando *et al*, 2007). Indeed, up to half of all human tumours have been found to be somehow driven by alterations in the mTOR pathway (Vivanco and Sawyers, 2002; Xu *et al*, 2004). In addition, it is also critical in some tumour microenvironment processes such as angiogenesis (Viñals *et al*, 1999; Guba *et al*, 2002; Hudson *et al*, 2002; Humar *et al*, 2002; Mayerhofer *et al*, 2002; Land and Tee, 2007). Therefore, targeting mTOR is a rational therapeutic approach in human cancer. Sirolimus, also known as rapamycin, was one of the first compounds able to inhibit mTOR (Wiederrecht *et al*, 1995). It is a macrolide that prevents the phosphorylation of S6 and 4EBP-1 and therefore their activation (Brown *et al*, 1994; Faivre *et al*, 2006). Some of its derivatives, namely everolimus, temsirolimus and ridaforolimus, have been successfully assessed in phase III trials in different malignancies (Hudes *et al*, 2007; Motzer *et al*, 2008; Yao *et al*, 2011; Baselga *et al*, 2012; Demetri *et al*, 2013).

Gemcitabine is a pyrimidine analogue that targets cells undergoing DNA synthesis and blocks progression of cells from G1 to S-phase (Elnaggar *et al*, 2012). It is currently used in a vast spectrum of tumours either alone or in combination thanks to its favourable toxicity profile (Gesto *et al*, 2012).

Combination of sirolimus with gemcitabine has been reported to increase apoptosis *in vitro* and enhance antitumoural activity *in vivo* on different epithelial tumours (Grünwald *et al*, 2002; Mondesire *et al*, 2004). Specifically in sarcomas, an *in vitro* study in leiomyosarcoma cell lines has shown that this combination has a synergic effect in extracellular-signal-regulated kinases (ERK 1/2) inhibition, producing a dramatic effect in cell cycle (Merimsky *et al*, 2007). However, no studies in xenograft sarcoma models have been published to date. Nevertheless, response in a patient affected by leiomyosarcoma has been reported (Merimsky, 2004) suggesting that this combination may have profound effects on these malignancies.

This phase I trial was designed to determine the recommended dose (RD), safety profile, pharmacokinetic (PK) parameters and pharmacodynamic activity of the combination of sirolimus and gemcitabine. Preclinical antitumour efficacy both *in vitro* and *in vivo* was also evaluated.

MATERIALS AND METHODS

Patient selection. To be enrolled in this study, patients had to meet the following eligibility criteria: diagnosis of advanced solid tumour that have progressed or are ineligible for standard treatment, no prior treatment with mTOR inhibitors or gemcitabine, Eastern Cooperative Oncology Group performance status (ECOG PS) 0–1, either measurable or evaluable disease and age $\geqslant 18$ and $\leqslant 70$ years. The upper limit of age was established due to the increased risk of toxicity often seen in some elderly patients. Adequate bone marrow, hepatic and renal function were mandatory and were defined as: absolute neutrophil count $\geqslant 1.5 \times 10^9 l^{-1}$, platelets $\geqslant 100 \times 10^9 l^{-1}$, bilirubin, aspartate aminotranferase (AST), alanine aminotransferase and creatinine $\leqslant 1.5\times$ upper limit of normal and creatinine clearance $\geqslant 60$ ml min^{-1}. Patients with a history of other previous malignancies diagnosed or treated in the past 5 years (except basal cell skin carcinoma, adenocarcinoma *in situ* of the uterine cervix and superficial bladder cancer) and known central nervous system metastases were considered ineligible. Other exclusion criteria were treatment with experimental drugs within 30 days prior, pregnancy or lactancy, presence of active infection or any concomitant serious disease.

All patients signed written informed consent and the study was conducted according to local and national ethical review board approval, the Declaration of Helsinki and standards of Good Clinical Practice.

Study design and drug dosage, escalation and administration. Sirolimus was administered as a continuous daily oral dose (2 or 5 mg) starting on day 2 of cycle 1 until progression or intolerance. Gemcitabine was administered intravenously at a fixed-dose rate of 10 mg m^{-2} min^{-1} on days 1 and 8 of each cycle. The duration of each cycle was 21 days. A maximum of six cycles of gemcitabine per patient were allowed. Single agent sirolimus was continued after six planned cycles of gemcitabine in the absence of progressive disease (PD) and good tolerance. Protocol was amended according to pharmacodynamic results and a new dose level was added (Table 1).

The trial was performed using a standard $3+3$ dose-escalation phase I design with cohorts of 3–6 patients. If less than one-third of patients at a dose level experienced a dose-limiting toxicity (DLT), dose escalation continued. If more than one-third but less than two-thirds of patients at a dose level had a DLT, three additional patients were enrolled at that same dose level. If two-thirds or more of patients at a dose level experienced a DLT, the dose was considered toxic and the next cohort of patients was included at the next lower dose level. Dose escalation within a patient was not permitted. Patients were withdrawn from study treatment when there was evidence of PD, unacceptable toxicity or consent withdrawal.

Routine clinical and laboratory assessments were conducted on a weekly basis. Toxicity was graded using the National Cancer Institute Common Toxicity Criteria version 3.0 (NCI-CTCAE v3.0). Dose-limiting toxicity was defined as any of the following within 3 weeks after the administration of the first cycle: absolute neutrophil count $<0.5 \times 10^9 l^{-1}$ over $\geqslant 5$ days or associated with fever $\geqslant 38.5^\circ$C, platelets $<50 \times 10^9 l^{-1}$, any grade 3–4 non-haematological toxicity (excluding nausea and vomiting non-refractory to antiemetic treatment) or skin rash grade 2 related to treatment and not controlled with support medication. Maximum tolerated dose (MTD) was defined as the highest dose level in which two or more patients experienced DLT. Next lower dose level was considered as RD.

In order to assess tumour response to treatment, thorax–abdomen–pelvis CT scans were performed every 6 weeks and Response Evaluation Criteria in Solid Tumors version 1.0 (RECIST v1.0) were used (Therasse *et al*, 2000).

Pharmacokinetics. Gemcitabine concentrations were measured at days 1 and 24 of the study and PK sampling was performed at 0.5, 1, 2.5, 4, 8, 10 and 24 h after the start of the infusion, which was ranged from 0.95 to 3.28 h.

Gemcitabine PK blood samples were collected in polypropylene tubes with EDTA, which contained tetrahydrouridine to inactivated gemcitabine degradation. After plasma separation by centrifugation, samples were stored at -80°C until analysis.

Table 1. Dose levels and dose-limiting toxicities (DLTs)

Dose level	Sirolimus (mg per 24 h) orally	Gemcitabine (mg m^{-2}) intravenously	DLT/ patients	Toxicity
1	2	800	0/3	
2	2	1000	1/6	Transaminitis G3
2.A	5	800	0/6	
3	5	1000	2/4	Thrombocytopenia G3 Thrombocytopenia G4

An Alliance 2695 (Milford, MA, USA) separations module and photodiode array detector, with Empower 2 software (Waters, Milford, MA, USA) to online data acquisition were used. Separation was performed on a Nova Pak C_{18} cartridge column, (Waters), which was maintained at 30°C. The mobile phases consisted of solutions of 5% (v/v) heptane sulfonic acid and methanol, and were delivered following a flow rate of 1 ml min^{-1}. Gemcitabine and its internal standard (2-desoxicitidina) were extracted from plasma samples by protein precipitation followed liquid–liquid extraction. This HPLC method was validated using quality control samples and standard of calibration obtained from spiked blank plasma samples with different concentrations of gemcitabine. Intra-assay and inter-day imprecision and accuracy was evaluated with the control samples plasma at three concentrations in four days and the values obtained were < 10% and 8%, respectively. The limit of quantification (LLOQ) was 200 $\mu g l^{-1}$ and measurements were linear from 200 to 20 000 $\mu g l^{-1}$ ($r^2 = 0.99$).

Sirolimus concentrations were measured at day 21 of the study before both gemcitabine and sirolimus dose administrations (predose concentrations). Sirolimus PK blood samples were collected into plasma tubes with EDTA-K_3 (Vacuette, Kremsmünster, Austria) and stored at −80°C until analysis. An Acquity UPLC integrated measurement system (Waters) was used. Separation was performed on a MassTraK TDM C_{18} cartridge column, 2.1 × 10 mm (Waters), which was maintained at 55°C. The mobile phases, consisted of solutions of ammonium acetate 2 mM and 0.1% (v/v) formic acid either in water or in methanol, and were delivered following a flow rate of 0.4 ml min^{-1}. Detection was carried out using an Acquity TQD tandem-quadrupole mass spectrometer equipped with a Z-spray electrospray ionisation source (Waters) operating in positive mode. Sirolimus and its internal standard ($[^{13}C_2D_4]$-everolimus) were detected in multiple reaction monitoring mode using mass-to-charge (m/z) transition of 931.9→864.4 and 981.9→914.4, respectively. The MassTrak immunosuppressants XE RUO kit provided by Waters was used. Intra-assay and inter-day coefficients of variation, accuracy and relative measurement errors ranged from 7.8% to 10.0%, 8.9% to 12.4%, −8.7% to −6.0% and −5.0% to 15.0%, respectively. The limit of quantification was 1.7 $\mu g l^{-1}$ and the measurement interval was linear between 1.7 and 31.1 $\mu g l^{-1}$ ($r^2 = 0.996$).

The population PK model development and simulations were performed with the nonlinear mixed-effects modelling (NONMEM) software, version 7.2 (ICON Development Solutions, Ellicott City, MD, USA) using the subroutine ADVAN3 TRANS4 (user-defined non-linear model). To statistically distinguish between nested models, the difference in the MOFV[8] (minimum objective function value) was used because this difference is approximately χ^2 distributed. A significance level of $P < 0.005$ that corresponded to a difference in MOFV of 7.879 for 1 degree of freedom was considered. Additionally, to the diagnostic plots used for evaluation during model building development with Xpose version 4.0 (Division of Pharmacokinetics and Drug Therapy, Uppsala University, Uppsala, Sweden), an internal validation was performed. The bootstrap method with replacement was used to assess the stability of the final model and to construct confidence intervals of PK parameters using the PsN-Toolkit (version 3.2.4; Division of Pharmacokinetics and Drug Therapy, Uppsala University, Uppsala, Sweden).

Pharmacodynamics. Paired skin biopsies were planned for every patient: at baseline and 21 days after first dose administration. In order to assess mTOR pathway inhibition, immunohistochemistry of phosphorylated S6 at Ser235/236 (pS6) #4858 was performed in formalin-fixed paraffin-embedded sections of skin samples using a 1 : 50 dilution of a rabbit polyclonal antibody (from Cell Signaling Technology, Danvers, MA, USA). Then, qualitative changes in the expression of pS6 were assessed.

***In vitro* study.** Two sarcoma cell lines acquired from Cell Lines Service (CLS, Eppelheim, Germany) were used to assess the *in vitro* efficacy of the treatment: SKLMS-1 and SW982 (leiomyosarcoma and synovial sarcoma, respectively). Both cell lines were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) and were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Cell proliferation assay. Sirolimus and gemcitabine were diluted in cell medium at 20 ng ml^{-1} and 100 nM, respectively and then cells were treated with both drugs separately, sequentially and in combination for 48 h. Dimethyl sulfoxide (DMSO) was added to cultures as control. Cell proliferation and cell death were determined by the trypan blue exclusion assay.

Western blot. SKLMS-1- and SW982-treated cells were lysed with radioimmunoprecipitation assay buffer containing protease inhibitors (1 mmol l^{-1} phenylmethylsulfonyl fluoride, 10 mg ml^{-1} aprotinin, and 10 mg ml^{-1} leupeptin) and the lysates were centrifuged at 13 000 × g, at 4°C, for 30 min. Lysate aliquots (50 μg) were resolved by 10% SDS–PAGE and transferred onto nitrocellulose membranes. After blocking with 5% skimmed milk in PBS containing 0.2% Tween 20 (Dallas, TX, USA) at room temperature for 1 h, membranes were incubated overnight at 4°C with the appropriate primary antibody (cleaved caspase 3 #9661, native S6 #2217, and pS6 #4858 from Cell Signaling Technology). Blots were then incubated at room temperature for 1 h with a horseradish peroxidase-conjugated secondary antibody and the peroxidase activity was detected by enhanced chemiluminescence (Pierce, Rockford, IL, USA) following the instructions of the manufacturer. Immunodetection of α-tubulin was used as a loading reference.

***In vivo* study.** An *in vivo* xenograft model was established by subcutaneous injection of 3.5×10^6 SKLMS-1 cells suspended in 100 μl of saline in athymic nude mice (BALB/cnu/nu) from Harlan (Indianapolis, IN, USA). Animal care and procedures were followed according to the Institutional Guidelines for the Care and Use of Laboratory Animals. Once tumours reached 100 mm^3, groups of five mice were treated with sirolimus 2.5 mg kg^{-1} and gemcitabine 60 mg kg^{-1} followed by sirolimus 2.5 mg kg^{-1} after 24 h. All treatments were administered in intraperitoneal manner for 2 weeks (sirolimus once daily and gemcitabine once weekly). An additional group of five mice were treated with DMSO as controls. Tumours were measured every 2 days with calipers, and toxicity was monitored by weight loss. Mice were killed once tumours reached 2500 mm^3 (or after manifestation of morbidity) and tumours were removed and stored in 4% paraformaldehyde. Immunohistochemistry was performed in formalin-fixed paraffin-embedded sections from tumour samples. Phosphorylated S6 was detected with a 1 : 50 dilution of a rabbit polyclonal antibody #4858 (from Cell Signaling Technology).

RESULTS

Patient characteristics. From June 2010 to September 2011, 19 patients were enrolled in a single centre. All patients were assessable for toxicity and efficacy. Demographics characteristics are shown in Table 2. All patients except one had received prior chemotherapy treatment. Median number of previous lines was 2.5 (range 0–6) and 7 (37%) patients had radiation therapy before enrolment in the study. A total of 77 cycles of the study regimen were administered. Median number of cycles per patient was 4 (range 1–6).

Safety. All 19 patients were evaluable for DLT. Initially, the three dose levels planned were evaluated. One patient experienced DLT consisting in grade 3 transaminitis at dose level 2 and two patients experienced DLT at dose level 3 consisting in grade 3

Table 2. Demographics and baseline characteristics

	Total patients ($n=19$)
Gender	
Male	7 (37%)
Female	12 (63%)
Age	
Median	51
Range	36–70
ECOG PS	
0	5 (26%)
1	14 (74%)
Tumour	
Colorectal	7
Gastric	3
Cervix	1
NSCLC	1
Poorly differentiated chondrosarcoma	1
Eccrine gland adeno	1
Renal clear cell	1
Thymoma	1
Adrenal carcinoma	1
Urothelial carcinoma	1
Anaplastic thyroidal	1
Previous treatment	
Lines of chemotherapy	
0	1 (5%)
1	3 (16%)
2	4 (21%)
>2	7 (37%)
Unknown	4 (21%)
Median	2.5 (range 0–6)
Radiotherapy	
Yes	7 (37%)
No	12 (63%)

Abbreviations: ECOG PS = Eastern Cooperative Oncology Group performance status; NSCLC = non-small cell lung cancer.

thrombocytopenia and grade 4 thrombocytopenia, respectively. Thus, MTD was reached at dose level 3. However, the pharmacodynamic analysis performed in the 13 patients treated at those dose levels revelled poor mTOR pathway inhibition at doses <5 mg of sirolimus. Therefore, an amendment was performed including a new dose level under the reached MTD consisting of sirolimus 5 mg and gemcitabine 800 mg m^{-2} (dose level 2.A). At this dose level, no DLT was observed and it was established as the RD (Table 1).

The majority of side effects reported were grade 1–2. The most commonly observed treatment-related events were haematological: anaemia (84%; $n=16$), neutropenia (68%; $n=13$) and thrombocytopenia (68%; $n=13$). The most frequent non-haematological toxicities were raised AST (58%; $n=11$), raised GGT (47%; $n=9$), hypercholesterolaemia (47%; $n=9$), anorexia (47%; $n=9$) and mucositis (42%; $n=8$). In general, toxicity was mild and easily manageable. No pulmonary toxicity was reported. Three patients required dose reduction of sirolimus, being grade 3 thrombocytopenia the reason in two cases and grade 2 fever in one case. Gemcitabine dose reduction was required in two patients due to grade 4 anaemia and grade 2 transaminitis, respectively. Toxicity is summarised in Table 3.

Pharmacokinetics and pharmacodynamics. Since gemcitabine is a drug with well-known activity against a large number of malignancies, we designed the study to determine whether the addition of sirolimus has any influence on its PK. Data from all 19 patients were used in the PK analysis. The effects of gender, age, weight (WGT), body surface area (BSA) and sirolimus through concentrations were assessed on gemcitabine PK at day 21. Demographic characteristics and sirolimus trough concentrations are summarised in Table 4. Correlation between WGT/BSA and height (HGT) was found.

The plasma concentration *vs* time profiles of gemcitabine at days 1 and 21 are displayed in Figure 1. It should be noted that quantifiable gemcitabine concentrations were found up to 2.5–4 h post administration in both occasions. The PK of gemcitabine after intravenous infusion of 10 mg m^{-2} min^{-1} in the target population was best described by a two-open-compartment model with first-order elimination. All recorded covariates were tested in the PK parameters, plasma clearance (CL) and central compartment distribution volume (Vc), with NONMEM, but no statistically significant relationship could be identified in any case. No statistically significant effect of anthropometric covariates (WGT, HGT and BSA) and age on the PK parameters was found ($P>0.05$) and no specific trends were observed between CL or Vc values and sirolimus concentrations (Supplementary Figure 1). The estimated PK parameters with final model (NONMEN) listed in Supplementary Table 1 were in agreement with those previously reported in the literature (Keith *et al*, 2003; Lin *et al*, 2004). Between-patient variability could be associated to CL (14.6%) and Vc (98.2%), meanwhile between-occasion variability could be to Vc (47.1%).

Immunohistochemistry of pS6 in patients' paired skin biopsies showed significant inhibition of mTOR at RD (Supplementary Figure 2). Weaker staining of pS6 was achieved with 5 mg (dose levels 2.A and 3) compared to 2 mg.

Efficacy. Two patients achieved partial response (PR): one patient at dose level 2.A (colon adenocarcinoma) and the other one at dose level 3 (uterine cervix cancer). Nine patients experienced stable disease (SD) as best response that lasted >12 weeks and in three cases, the duration of the stabilisation was at least 6 months.

In vitro study results

Cell proliferation assay results. Both cell lines were sensitive to gemcitabine and sirolimus. Interestingly, higher cell death rate was observed in both cell lines with the sequential treatment administering first gemcitabine and 24 h later sirolimus than with the inverse order or with the administration of both drugs at the same time (data not shown).

Western blot results. We used cleaved caspase 3 as apoptosis marker to assess the *in vitro* efficacy of the combination. Results showed that the greatest activation of apoptosis was achieved with the sequential treatment administering gemcitabine first followed by sirolimus 24 h later (Figure 2A).

We assessed by western blot phosphorylation of S6 as a marker of mTOR activity. Although the non-phosphorylated forms had no relevant changes with the treatment, pS6 was highly induced when cells were treated with gemcitabine alone. This induction was clearly reversed when sirolimus was added (Figure 2B).

***In vivo* study results.** Xenograft model was established using SKLMS-1 cells. According to *in vitro* results, treatment was administered in a sequential fashion (first gemcitabine and 24 h later sirolimus). Tumour growth was strongly inhibited with the sequential combination of the two drugs compared to Control and to each drug alone (Figure 3).

Table 3. Toxicity

	Dose level 1 ($n=3$)		Dose level 2 ($n=6$)		Dose level 2.A ($n=6$)		Dose level 3 ($n=4$)		Total ($n=19$) All grades		Total ($n=19$) Grade 3–4	
Toxicity	All grades	Grade 3–4	All grades	Grade 3–4	All grades	Grade 3–4	All grades	Grade 3–4	*n*	%	*n*	%
Anorexia	2		2		3		2		9	47		
Mucositis	2		2		3		1		8	42		
Fever			3		3		1		7	37		
Nausea/vomiting	1		3		2		1		7	37		
Fatigue			3		3				6	32		
Rash	2		3				1		6	32		
Diarrhoea					1		2		3	16		
Anaemia	2		4		6	1	4		16	84	1	5
Neutropenia	2	1	3	1	5	3	3	1	13	68	6	32
Thrombocytopenia	1	1	4		5		3	2	13	68	3	16
Leukopenia	1		1		3		3		8	42		
Raised AST	1		4	1	4		2		11	58	1	5
Raised GGT	3	1	2	1	4				9	47	2	11
Hypercholesterolaemia	1		4	1	3		1		9	47	1	5
Raised ALT	1		3	2	2		1		7	37	2	11
Hyperglycaemia			1		2		2		5	26		
Raised creatinine			1						1	5		

Abbreviations: ALT = alanine aminotransferase; AST = aspartate aminotranferase; GGT = gamma-glutamyl transferase. Toxicities reported at any time from first treatment administration to 30 days of last treatment administration are included.

Table 4. Demographic characteristics of patients of the study population

Patients' characteristics	Mean (RSE%)	Median	Minimum	Maximum
Patients (*n*)	19	—	—	—
Age (years)	54.1 (18.6)	54.5	36	70
Gender (*n*), male	13	—	—	—
Female	6	—	—	—
Height (cm)	166.9 (10.6)	167	151	184
Body weight (kg)	73.0 (22.0)	75	44.2	107
Body surface area (m^2)	1.81 (13.22)	1.90	1.40	2.30
Sirolimus concentrations ($\mu g\ l^{-1}$)	9.05 (7.78)	7.60	0.90	28.50

Abbreviation: RSE% = relative standard error.

Immunohistochemistry results. Strong pS6 staining in tumours treated with gemcitabine alone was observed. In contrast, that staining was dramatically absent in tumours treated with the combination, indicating that the addition of sirolimus is able to reverse pS6 induction also *in vivo* (Figure 4).

DISCUSSION

This study demonstrates that the combination of sirolimus and gemcitabine is feasible and safe, allowing administration of active doses of both agents and achieving mTOR pathway inhibition even in heavily pretreated patients. The most common adverse events registered were haematological, but they were generally mild and easily manageable. Other mild toxicities observed were raised liver enzymes, hypercholesterolaemia, anorexia and mucositis, all of them usually related to either sirolimus or gemcitabine in monotherapy, but modifications in the treatment schedule or dose were not necessary in almost any case. Furthermore, the toxicity profile showed no synergistic effects in these adverse events with the combination of the two drugs. Transaminitis grade 3 and thrombocytopenia grades 3 and 4 where the DLTs found, all of them are relatively common and expected in patients treated with gemcitabine. No unexpected toxicity appeared with the treatment. Moreover, PK showed no effects of sirolimus concentrations on gemcitabine clearance. This favourable profile leads us to

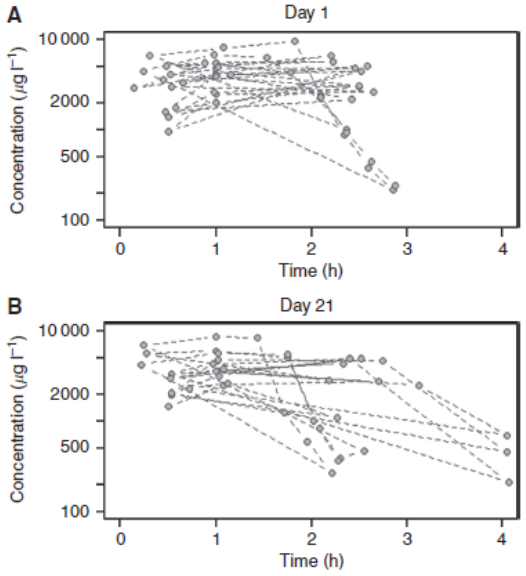

Figure 1. (**A**) Observed gemcitabine plasma concentrations ($\mu g\,l^{-1}$) *vs* time (h) after intravenous infusion of 10 mg m^{-2} min^{-1} on day 1. (**B**) Observed gemcitabine plasma concentrations ($\mu g\,l^{-1}$) *vs* time (h) after intravenous infusion of 10 mg m^{-2} min^{-1} on day 21.

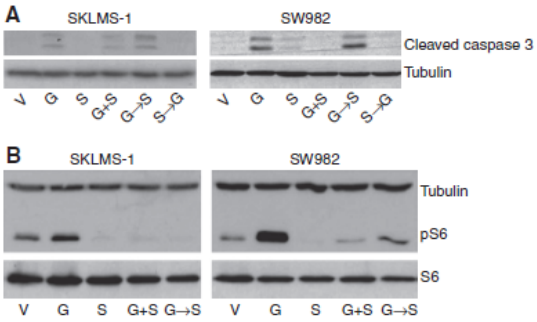

Figure 2. (**A**) Western blot cleaved caspase 3. The greatest cleavage of caspase 3 was achieved when treatment was administered in a sequential manner: first gemcitabine followed by sirolimus 24 h later. (**B**) Western blot pS6 and S6. The activation of S6 observed when cells were treated with gemcitabine alone was reversed with the addition of sirolimus. G = gemcitabine; S = sirolimus; V = control.

recommend dose level 2.A (sirolimus 5 mg per 24 h plus gemcitabine 800 mg m^{-2}) as the optimal dose due to its well-proved safety record.

In addition, the preclinical study also showed encouraging results. Thus, the *in vitro* study showed that caspase 3 cleavage was more evident when cells were treated sequentially (gemcitabine before sirolimus) than administering both drugs simultaneously. Therefore, a clear pro-apoptotic induction as a result of this combination is responsible for the dramatic effect on tumour survival. Sequential administration of drugs, including sirolimus, as a cancer therapeutic strategy has been used elsewhere (Iacovelli *et al*, 2013; Rosa *et al*, 2013). mTOR inhibition results in downregulation of several antiapoptotic proteins such as Bcl-xL

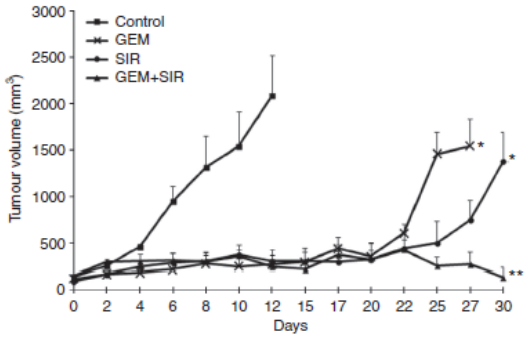

Figure 3. SKLMS-1 xenograft tumour growth. *t*-Test: *$P \leqslant 0.03$; **$P \leqslant 0.0001$. Leiomyosarcoma xenograft tumour growth was strongly inhibited by the combination treatment. GEM = gemcitabine; SIR = sirolimus.

and Mcl-1 (Tirado *et al*, 2005; Faber *et al*, 2014). Thus, sirolimus addition sequentially after gemcitabine may prevent resistance to this drug through antiapoptotic pathway activation. In agreement with this hypothesis, several reports demonstrate that inhibition of antiapoptotic bcl-2 family members sensitises tumour cells to gemcitabine (Schniewind *et al*, 2004; Zhang *et al*, 2011). In contrast, one of the main effects of mTOR inhibition is G1 arrest (Carew *et al*, 2011) that makes cells less prone to be damaged by gemcitabine. This hypothesis is being currently tested in the laboratory. On the other hand, we found both *in vitro* and *in vivo* that S6 was activated when cells were treated with gemcitabine alone but such activation dramatically reversed when sirolimus was added, correlating with the efficacy of the combinatory treatment. These interesting data suggest hyperactivation of mTOR pathway as a cellular mechanism of defence triggered by gemcitabine that can be reversed with the addition of sirolimus. This brand new finding opens an exciting line of investigation worth exploring. Furthermore, xenograft tumour growth was dramatically reduced with the combined treatment and pharmacodynamic analysis showed an effective mTOR inhibition at RD, making this therapeutic strategy even more promising.

Combination of an mTOR inhibitor with conventional chemotherapy with gemcitabine could be a way to improve the efficacy of either of the agents alone in different tumour types such as pancreatic cancer, renal cell cancer or sarcomas. Specifically, in sarcomas, positive results with mTOR inhibitors have been reported. Thus, sirolimus and its derived temsirolimus have shown activity in perivascular epithelioid cell tumours (PEComas), a specific subtype of mesenchymal tumour (Italiano *et al*, 2010; Wagner *et al*, 2010). Moreover, it has been recently published in a positive phase III trial in sarcomas with the mTOR inhibitor ridaforolimus. This double-blind, placebo-controlled phase III trial randomised 702 sarcoma patients who had achieved CR, PR, or SD after 1, 2, or 3 lines of chemotherapy to receive placebo or ridaforolimus as maintenance treatment. Ridaforolimus showed signs of activity, inducing a mean 1.3% decrease in target lesion size *vs* a 10.3% increase with placebo. In addition, it achieved a statistically significant improvement in PFS compared to placebo in both independent and per investigator assessment. However, the magnitude of that improvement was very modest (median PFS 17.7 weeks *vs* 14.6 weeks per independent review; Demetri *et al*, 2013). These results, positive but excessively limited, suggest some important conclusions: mTOR inhibitors are active in sarcomas but the best therapeutic strategy is still unknown. Thereafter, combination treatments with mTOR inhibitors and cytotoxic drugs

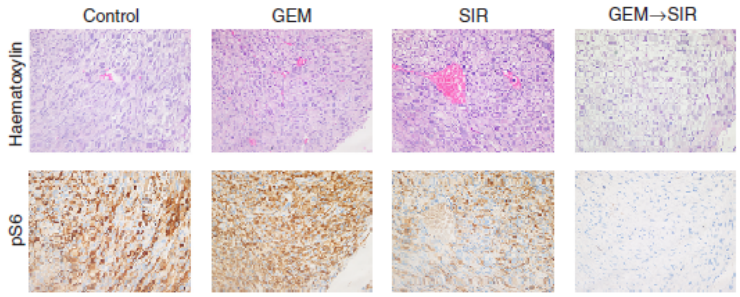

Figure 4. Immunohistochemistry of pS6 in leiomyosarcoma xenograft samples. Sirolimus is able to reverse the hyperactivation of the mTOR pathway caused by gemcitabine in leiomyosarcoma xenografts. GEM = gemcitabine; SIR = sirolimus.

(like the one assessed in this study) are a promising alternative that deserve further investigation.

In conclusion, this phase I trial of the combination of sirolimus and gemcitabine demonstrated that this regimen is feasible and safe. Moreover, it showed signs of activity both *in vitro* and *in vivo*. In addition, mTOR inhibition was achieved at RD and PK analysis showed no influence of sirolimus on gemcitabine clearance. Further studies to assess the activity of this combination are warranted and a phase II trial in sarcomas is ongoing (ClinicalTrials.gov identifier NCT01684449).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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7. Discussion

Our results support that the combination of targeted therapies with cytotoxic drugs is a safe and active therapeutic strategy in sarcomas. Our preclinical studies demonstrated that combining antiangiogenic compounds such as sorafenib with cytotoxic agents such as ifosfamide has higher activity than each drug alone. Similar results in *in vitro* and in *in vivo* sarcoma models were observed with the mTOR inhibitor sirolimus combined with the classic chemotherapy drug gemcitabine. Moreover, we showed that the mTOR pathway was activated as a result of gemcitabine administration, presumably representing a mechanism of resistance to chemotherapy. Interestingly, this effect was reversed with sirolimus. In the clinical setting, we demonstrated that the combination of sorafenib plus ifosfamide and sirolimus plus gemcitabine are feasible and safe regimes, allowing the administration of active doses of each agent. Early signs of clinical activity were also seen.

In our first study, we demonstrated that blocking tumor angiogenesis with the TKI sorafenib combined with the chemotherapy active in sarcomas ifosfamide is a tolerable therapeutic strategy (204). Our combinatory regimen was supported by our own preclinical research which preceded the clinical trial. Using cell lines from 2 of the most common sarcoma subtypes, leiomyosarcoma and synovial sarcoma, we found a higher activity of the combination on both cell lines compared to each drug alone. Cell proliferation and death experiments showed an additive effect *in vitro* of the treatment which encouraged us to conduct the phase I clinical trial to confirm whether such combinatory strategy was safe in sarcoma patients. In the clinical study, we found

that sorafenib and ifosfamide can be safely administered combined. Moreover, active doses of both agents (sorafenib 400 mg bid and ifosfamide 6 g/m2) can be delivered without significant toxicity since the safety profile of the combination is very similar to each drug alone, showing no synergistic effect in toxicity.

The dose limiting toxicities (DLTs) observed were fatigue, vomiting and encephalopathy (one each). Fatigue is a very frequent symptom in advanced cancer, being present in almost every patient. In a study of 1000 patients in an American palliative care programme, fatigue, weakness, and lack of energy were 3 of the 5 most frequently reported symptoms with a prevalence of 84%, 66%, and 61%, respectively (205). It can be caused by multiple factors including the own treatment and/or cancer-induced situations such as excessive catabolic state (206). In any case, it is not a life-threatening adverse event and is usually manageable. The other 2 DLTs observed were vomiting and encephalopathy. These are frequent side effects classically associated with ifosfamide. Adequate anti-emetic prophylaxis and treatment of encephalopathy with methylene blue (as described previously) are easy manoeuvres so these 2 adverse events should not preclude treatment with ifosfamide. Interestingly, other ifosfamide-associated important side effects such as hemorrhagic cystitis or renal insufficiency were not observed, indicating no toxicity enhancement with concomitant sorafenib treatment. Other toxicity reported such as mucositis, diarrhea, skin rash, hand-foot syndrome or hematological toxicity are side effects expected with sorafenib or ifosfamide and they did not lead to substantial treatment schedule modifications. It is worth mention that no unexpected toxicity was found. Cardiac toxicity was a safety problem in previous clinical trials evaluating combinations of antiangiogenic agents and chemotherapy in sarcoma (76) but not in our study. One of the most common and

specific side effects of the angiogenesis inhibitors is cardiovascular toxicity. It appears in around 30% of cases and it usually manifests in the form of hypertension, although ischemic cardiac disease is not a rare event (207). Doxorubicin, the most active drug in sarcomas to date together with ifosfamide, has also the potential to induce cardiac damage leading to life-threatening cardiomyopathy and heart failure (208). The estimated overall incidence of congestive cardiac failure is 3%–5% at a total cumulative doxorubicin dose of 400 mg/m2, increasing to 7%–26% at 550 mg/m2 and up to 48% at 700 mg/m2 (209). Therefore, combining doxorubicin with antiangiogenic agents would presumably have led to serious cardiac toxicity as occurred in the already described D'Adamo et al. study of doxorubicin plus bevacizumab (76). That presumption, together with the results of our preclinical studies, made us choose ifosfamide as the companion chemotherapeutic agent of our antiangiogenic plus chemotherapy regimen. As reviewed earlier, ifosfamide is similar to doxorubicin in terms of efficacy in sarcomas but it is not related to the development of cardiac toxicity so we hypothesized that we could achieve maximum efficacy without significant toxicity in combination with sorafenib. The favourable safety profile observed in our study confirmed our hypothesis. In addition, PK analysis showed no decrease in sorafenib and ifosfamide plasma levels with concomitant administration of both agents. In fact, we found that the active ifosfamide metabolite 4-hydroxy-ifosfamide increases with concomitant sorafenib administration. These results should be interpreted cautiously since they are based on only a limited number of plasma samples and they might have been influenced by a high variability but they suggest that sorafenib could enhance ifosfamide activity. This is an interesting feature of our combinatory regimen because a phase I trial previously reported found that ifosfamide

induces decreased sunitinib blood levels because of CYP3A induction. In addition, a synergic effect in toxicity with high neutropenia rates was observed leading to a final tolerable dose of sunitinib too low to be considered clinically relevant (210). Therefore, our study demonstrates that sorafenib is a better antiangiogenic agent to combine with ifosfamide than sunitinib in terms of PK and toxicity.

Moreover, with the limitations of a phase I study in terms of evaluating clinical efficacy, the combination of sorafenib plus ifosfamide showed promising activity results. Although no PR was reported, 72.7% patients achieved SD for more than 12 weeks which is encouraging for such a heterogeneous and heavily pretreated population of sarcoma patients. In addition, PFS data met the criteria proposed by the EORTC for the development of drugs in sarcomas (211), making the combination of ifosfamide and sorafenib attractive for further evaluation. Indeed, at least two phase II studies have recently assessed this therapeutic strategy in sarcomas. One of them, conducted by our group, is the continuation of the study herein reported. In that phase II trial, 35 patients with advanced sarcomas who had received prior doxorubicin were treated with the combination of sorafenib and ifosfamide at the dose and schedule recommended by our phase I study (sorafenib 400 mg bid continuously plus ifosfamide 6 g/m2 every 3 weeks). Primary endpoint was PFS rate at 3 months, which was found to be 67%. Median PFS and median OS were 4.8 months and 16.2 months, respectively. PR was achieved in 17% of patients. These results are superior to what is expected with ifosfamide alone, supporting the interest of our combinatory regimen (212). The other study with sorafenib and ifosfamide conducted in sarcomas, specifically addressed to patients in the neoadjuvant setting, has been concluded but no results have been reported yet (NCT00880542).

Regarding our second article, the results observed in the studies we conducted in *in vitro* and *in vivo* models of sarcoma with sirolimus plus gemcitabine encouraged us to further develop the combination (213). Using the same cell lines of leiomyosarcoma and synovial sarcoma that we used in our first article, our cell death studies showed that higher cell death rate and activation of apoptosis was achieved when cells were treated sequentially (gemcitabine before sirolimus) compared with each drug alone and with concomitant administration. This observation might be at least partially explained by previous studies conducted in an *in vitro* model of another type of sarcoma: Desmoplastic Small Round Cell Tumors (DSRCT). In such studies, it was found that sirolimus induced apoptosis in a DSRCT cell line by increasing the Bax : Bcl-xL ratio through concomitant downregulation of the antiapoptotic protein Bcl-xL and upregulation of the proapoptotic protein Bax, both at the post-transcriptional level. Interestingly, it was also observed that sirolimus induced apoptosis by preventing the degradation of the Bax protein by the proteasome in a process independent of mTOR inhibition (214). Furthermore, in a non-sarcoma malignancy such as CRC, mTOR inhibition also has been found to enhance apoptosis by downregulation of Mcl-1, another antiapoptotic protein (215). In addition, it has been described that inhibition of antiapoptotic bcl-2 proteins makes tumor cells more sensitive to gemcitabine (216, 217). Overall, data support that gemcitabine administration before sirolimus may prevent resistance by antiapoptotic pathway activation to the chemotherapy agent, which agrees with our *in vitro* results in leiomyosarcoma and synovial sarcoma. *In vivo*, we observed that tumor growth in our leiomyosarcoma xenograft model was dramatically inhibited with the same sequential combination, achieving much better

anti-tumor activity than with each drug in monotherapy. Moreover, we reported both *in vitro* and *in vivo* the striking finding that the mTOR pathway was activated as a result of the treatment with gemcitabine, which might represent a cellular mechanism of resistance to the aggression of the cytotoxic agent. Interestingly, the activation of the mTOR pathway was inhibited when sirolimus was added, correlating with the superior efficacy of the sequential treatment. This novel finding is a potential actionable mechanism of resistance to chemotherapy never reported before in sarcomas which merits further investigation.

Such promising preclinical results led us to assess the toxicity profile of the combination of sirolimus plus gemcitabine in humans. Increased liver enzymes and thrombocytopenia, expected side effects of gemcitabine, were the DLTs found. Hematological toxicity, hypercholesterolemia, anorexia and mucositis were other adverse events observed, all of them frequently seen with gemcitabine or sirolimus. Overall, the regimen was well tolerated, with no unexpected adverse events and no synergistic effects in toxicity observed with the combination of both agents. Furthermore, modifications in the treatment schedule or dose were not necessary in almost any case. We also demonstrated with paired skin biopsies that significant inhibition of the mTOR pathway was achieved at the recommended dose of sirolimus 5 mg every 24 hour plus gemcitabine 800 mg/m2 on days 1 and 8 every 3 weeks, confirming the proof-of-mechanism of our combinatory schedule. In addition, we studied if sirolimus had any influence on the already well-known gemcitabine PK and results showed no effects of sirolimus concentrations on gemcitabine clearance. PK parameters were in agreement with those previously reported in the literature (218, 219), further supporting the suitability of our combination.

In light of the favourable tolerability observed in the phase I study, together with the PK and pharmacodynamics analyses results, our group conducted a single-arm phase II clinical trial in patients affected by advanced soft tissue sarcomas. Twenty-eight heavily pretreated patients were enrolled and received our proposed combination of sirolimus plus gemcitabine. Preliminary results presented at the American Society of Clinical Oncology (ASCO) Annual Meeting 2014 showed that PFR rates at 3 and 6 months were 44% and 20%, respectively, meeting the primary endpoint of the study as recommended by the EORTC for sarcomas (211). Median PFS observed was 1.85 months and median OS was 9.1 months (220). These efficacy results might have been hampered by the heterogeneous heavily pretreated population enrolled in the study, although they succeeded in meeting the trial prespecified criteria for positivity. The manuscript with the definitive results is currently under elaboration by our group. On the other hand, the Spanish Group for Research on Sarcomas (GEIS) has evaluated this combinatory treatment also in osteosarcoma, the most frequent bone sarcoma. In a single-arm phase II study, 36 patients with advanced osteosarcoma received the combination of sirolimus and gemcitabine in second line of treatment at the dose and schedule recommended by our study. There were 2 PR (6%), 13 SD (39%) and 18 progressive disease (PD) (54%) as best responses. PFS rate at 4 months, the main endpoint of the study, was 44% and at 6 months it was 28%. Median PFS and OS were 2.3 months and 11.2 months, respectively. Similarly to our study, toxicity profile reported was mild and the trial was considered positive according to the prespecified PFS rate criteria defined by the authors, which further supports our combinatory strategy (221).

Both combinatory regimes here evaluated are promising therapeutic strategies but they also have some limitations. Probably, the most important one is the absence of biomarkers to guide the selection of patients more likely to benefit from such treatments. Identification of predictive biomarkers is an outstanding issue in sarcomas. Apart from GIST, in which constitutive activation of KIT receptor by mutations (222) is a well-established predictive factor for response to KIT inhibitors (223), molecular characterization of sarcomas is still not truly relevant in treatment decision. It is well-known that different histologic subtypes are more likely to respond to certain drugs than others (18). However, the underlying molecular characteristics behind that special sensitivity to some therapies depending on the histology are still not fully understood. Initiatives such as The Cancer Genome Atlas (TCGA) project (224) have not shed any light on this field yet. This collaborative effort conducted by the National Cancer Institute (NCI) and the National Human Genome Research Institute (NHGRI) aims to generate comprehensive, multi-dimensional maps of the key genomic changes in major types and subtypes of cancer (225). Molecular characterization of a large number of tumor types has already been reported (226). However, only preliminary results are available for sarcomas. Those provisional results, presented at the ASCO Annual Meeting 2015, were extracted from matched tumor-normal tissue whole exome sequencing of 242 sarcoma patients in which recurrent mutations were analyzed for statistical significance and potential oncologic relevance. The statistically significant recurrent mutations reported included recurrent inactivating mutations in 3 well-established tumor suppressors, *TP53*, *ATRX* and *RB1*, found in 27.3%, 8.7% and 6.2% of the total cohort respectively. In the analyses of specific histological subtypes, differential mutational frequency of those genes was noted (227). The reported

alterations, although oncogenic, are not currently 'druggable' since they affect tumor suppressor genes and most targeted therapies developed to date are inhibitors of oncogenes. This is therapeutic challenge because carcinogenesis is a multistep process attributable to gain-of-function mutations in oncogenes and, more frequently, to loss-of-function mutations in tumor suppressor genes (228). Efforts in this area are therefore needed if we are to improve the survival of patients affected by sarcomas.

Furthermore, given that the activity of our combinatory regimes has been confirmed in single-arm phase II clinical trials, the next step should be their comparison with other therapies in randomized studies to more accurately determine their role in the treatment of sarcomas. Unlike other malignancies (229), there are no approved treatments with combinatory regimes of targeted therapies and cytotoxic drugs for sarcomas to date. The place of novel approaches such as ours in the therapeutic algorithms of sarcomas needs therefore to be defined. Our schedule of sorafenib plus ifosfamide should be assessed in comparison with treatments like single agent ifosfamide or pazopanib, for instance, to confirm whether our combinatory strategy is superior to already approved drugs. Also, head to head trials comparing our sirolimus plus gemcitabine regimen with gemcitabine alone or with gemcitabine-based schedules are the next sensible step to take in its clinical development.

To conclude, in a group of malignancies with scarce treatment options such as sarcomas, any therapeutic approach with a solid preclinical rationale and encouraging signs of activity such as the combination of sorafenib plus ifosfamide and sirolimus plus

gemcitabine should be exhaustively explored. Together with other novel therapeutic strategies, namely sequential schedules or immunotherapy agents, our proposed combinatory regimes might help in achieving the final goal of improving the poor prognosis of patients affected by sarcomas without an impact in their quality of life. This is an urgent need that will hopefully be satisfied within the next few years.

8. Conclusions

- In *in vitro* models of leiomyosarcoma and synovial sarcoma, the combination of sorafenib and ifosfamide showed higher activity than each drug alone. An additive effect was observed.

- In the clinical setting, the combination of sorafenib and ifosfamide is a feasible and safe treatment, allowing administration of active doses of both agents without synergistic effects in toxicity.

- PK analyses showed no decrease in sorafenib and ifosfamide plasma levels with concomitant administration. Concentration of ifosfamide active metabolite 4-hydroxy-ifosfamide levels augment with concomitant sorafenib, suggesting that sorafenib could enhance ifosfamide effect without increasing toxicity.

- Early signs of clinical activity in heavily pre-treated patients affected by advanced sarcoma were seen in our phase I study with the combination of sorafenib and ifosfamide. Those findings have been confirmed in a single-arm phase II clinical trial with the same regimen conducted by our group.

- Both *in vitro* and *in vivo*, gemcitabine followed by sirolimus achieved higher anti-tumor activity than each drug administered alone or concomitantly in leiomyosarcoma and synovial sarcoma models.

- Both in *in vitro* and *in vivo* models of leiomyosarcoma and synovial sarcoma, the mTOR pathway was activated as a result of the treatment with gemcitabine, which might represent a mechanism of resistance to the chemotherapy. This activation was inhibited when sirolimus was added. This is a novel finding in sarcomas and a potential actionable mechanism of resistance to chemotherapy.

- In our xenograft model of leiomyosarcoma, we observed that tumor growth was dramatically inhibited with the same sequential combination of sirolimus and gemcitabine, achieving much better anti-tumor activity than with each drug in monotherapy.

- In the clinical setting, the combination of sirolimus and gemcitabine is a feasible and safe treatment, allowing administration of active doses of both agents without

synergistic effects in toxicity. Pharmacodynamics analyses showed inhibition of the mTOR pathway at the recommended dose and PK analyses showed no effects of sirolimus concentrations on gemcitabine clearance.

- In a phase II study conducted by our group with the recommended dose of the combination of sirolimus and gemcitabine found in our phase I trial, clinical activity in heavily pre-treated advanced sarcoma patients was seen.

- The combinations of sorafenib plus ifosfamide and sirolimus plus gemcitabine are feasible and safe regimes with clinical activity in sarcomas. Further investigation is needed.

9. Resumen en castellano

Combinación de agentes citotóxicos y terapias dirigidas para el tratamiento de los sarcomas avanzados: justificación preclínica y desarrollo clínico precoz

9.1 Introducción

El término “sarcoma” comprende más de 50 tumores diferentes que tienen en común su origen mesenquimal. A pesar de sus diferentes características, en la mayoría de los casos el pronóstico de los pacientes afectos por estas neoplasias es malo y esto es principalmente debido a la ausencia de tratamientos efectivos. Así, las drogas más activas en sarcomas hasta la fecha (antraciclinas e ifosfamida) sólo alcanzan una tasa de respuestas (TR) de 20-30% o incluso más bajas dependiendo de las series (23, 24). Se han conseguido TR más altas con la administración concomitante de ambos fármacos pero este régimen no está exento de toxicidades potencialmente serias, limitando su uso a una población seleccionada de pacientes con muy buen estado general que no es lo más común en la práctica clínica habitual (27). La gemcitabina es otro agente citotóxico activo en sarcomas aunque su eficacia en monoterapia es modesta (33). En los últimos años, las combinaciones de gemcitabina con otras drogas citotóxicas han conseguido buenos resultados de eficacia con un perfil tolerable de efectos secundarios (37, 39, 40). En cualquier caso, alrededor de la mitad de todos los pacientes diagnosticados de sarcoma desarrollarán metástasis en algún momento del curso de su enfermedad incluso si son diagnosticados en estadíos tempranos (8). Este comportamiento tan agresivo, junto con los pobres resultados conseguidos con los

fármacos disponibles actualmente, supone que los pacientes con sarcoma avanzado tengan una supervivencia global (SG) de alrededor de 1 año (9). Por lo tanto, es obligatorio identificar nuevas estrategias terapéuticas activas capaces de mejorar el pronóstico de los pacientes afectados por este grupo de enfermedades. Muchos esfuerzos han sido llevados a cabo en este sentido durante décadas pero no se han conseguido grandes resultados (11). En los últimos años, varias terapias dirigidas se han desarrollado en oncología (12) pero, desafortunadamente, sólo unos pocos de esos nuevos compuestos han alcanzado resultados positivos en sarcomas, siendo el tumor del estroma gastro-intestinal (GIST) el ejemplo más importante (13, 14). De hecho, sólo dos estudios fase III con nuevas terapias dirigidas en sarcomas aparte de GIST han arrojado resultados positivos hasta la fecha, indicando que es necesario seguir investigando (15, 16). Es más, la revolución de la inmunoterapia para el tratamiento del cáncer que estamos viviendo en estos momentos no ha afectado aún a los sarcomas. Con la excepción de la terapia celular con linfocitos T dirigidos contra el antígeno NY-ESO en sarcoma sinovial (17), los agentes inmunoterápicos aún no han conseguido resultados clínicamente significativos en sarcomas (18). Uno de los aspectos que clásicamente ha hecho difícil el encontrar nuevas drogas activas en sarcomas es la ausencia de dianas terapéuticas moleculares. Sin embargo, grandes avances se han hecho recientemente en el conocimiento de la biología molecular de los sarcomas (19) así como de sus vías de señalización intracelular (20). Por ejemplo, la vía de la angiogénesis o de la diana de rapamicina en células de mamífero (mTOR), entre otras, han sido reconocidas como de gran importancia en la patogénesis de los sarcomas (21, 22). El desarrollo de estrategias terapéuticas que inhiban dichas vías, en

combinación con agentes citotóxicos activos que potencien su eficacia, es un área de investigación que merece ser explorada.

9.2 Hipótesis y objetivos

9.2.1 Hipótesis

La inhibición de la angiogénesis y de la vía de mTOR en sarcomas en combinación con agentes citotóxicos activos potencia la actividad anti tumoral de cada una de las estrategias terapéuticas por separado sin toxicidad significativa.

9.2.2 Objetivos primarios

- Evaluar la seguridad y determinar la dosis recomendada para el desarrollo clínico en sarcomas de dos combinaciones de agentes citotóxicos y terapias dirigidas: ifosfamida más sorafenib y gemcitabina más sirolimus.

- Evaluar de forma preliminar las señales de actividad clínica de las combinaciones de ifosfamida más sorafenib y gemcitabina más sirolimus en sarcomas avanzados y en otros tumores sólidos.

9.2.3 Objetivos secundarios

- Evaluar las propiedades farmacocinéticas (PK) de las combinaciones de ifosfamida más sorafenib y gemcitabina más sirolimus.

- Evaluar las propiedades farmacodinámicas de la combinación de gemcitabina más sirolimus.

- Evaluar la actividad anti tumoral de la combinación de ifosfamida más sorafenib en modelos *in vitro* de sarcoma.

- Evaluar la actividad anti tumoral y los efectos en la vía de mTOR de la combinación de gemcitabina más sirolimus en modelos *in vitro* e *in vivo* de sarcoma.

9.3 Artículos

9.3.1 Artículo 1: Ensayo clínico fase I de sorafenib en combinación con ifosfamida en pacientes con sarcoma avanzado: un estudio del Grupo Español de Investigación en Sarcomas (GEIS)

Este ensayo clínico fase I evaluó la seguridad, la farmacocinética, la toxicidad limitante de dosis, la dosis máxima tolerada y la dosis recomendada de la combinación de sorafenib más ifosfamida en pacientes con sarcoma avanzado.

Métodos: Doce pacientes con sarcoma (9 sarcomas de tejidos blandos y 3 sarcomas óseos) fueron tratados con sorafenib más ifosfamida a dosis iniciales de 200 mg bid y 6 g/m2 respectivamente. Se utilizó un diseño 3 + 3 de escalada de dosis con cohortes de 3-6 pacientes. También se realizó un estudio *in vitro* para evaluar la eficacia preclínica de la combinación.

Resultados: Se observaron 3 toxicidades limitantes de dosis: fatiga grado 4 con sorafenib 400 mg bid más ifosfamida 6 g/m2 y encefalopatía y émesis grado 3 con sorafenib 400 mg bid más ifosfamida 7,5 g/m2. También se observaron otras toxicidades como diarrea, síndrome mano-pie, mucositis, neutropenia, erupción cutánea y plaquetopenia. No se observaron efectos relevantes en la PK de sorafenib

pero sí un aumento de 4-hidroxifosfamida, el metabolito activo de la ifosfamida. Ocho pacientes consiguieron estabilización de su enfermedad durante más de 12 semanas. *In vitro* se observó un efecto aditivo.

Conclusiones: La dosis recomendada fue sorafenib 400 mg bid más ifosfamida 6 g/m2, un esquema que permite la administración de dosis activas de ambos fármacos. También se observaron signos preliminares de actividad antitumoral.

9.3.2 Artículo 2: Ensayo clínico fase I y evaluación de la eficacia preclínica del inhibidor de mTOR sirolimus más gemcitabina en pacientes con tumores sólidos avanzados

Llevamos a cabo un ensayo clínico fase I en pacientes con tumores sólidos avanzados para identificar la dosis recomendada, evaluar la PK, la actividad farmacodinámica y la eficacia antitumoral preclínica de la combinación de sirolimus y gemcitabina.

Métodos: Diecinueve pacientes fueron tratados con sirolimus 2 o 5mg al día y gemcitabina 800 o 1000mg/m2 en días 1 y 8. La escalada de dosis dependió de la tasa de toxicidad limitante de dosis durante las primeras 3 semanas de tratamiento. Se realizaron biopsias cutáneas pareadas para evaluar la fosforilación de S6 (pS6) como marcador de la inhibición de la vía de mTOR. También se realizó una evaluación de la PK y de la eficacia preclínica de la combinación utilizando 2 líneas celulares diferentes de sarcomas y modelos murinos de leiomiosarcoma.

Resultados: Tres toxicidades limitantes de dosis fueron observadas: elevación de transaminasas grado 3, plaquetopenia grado 3 y plaquetopenia grado 4. Los efectos

adversos más comúnmente encontrados fueron anemia, neutropenia, plaquetopenia y elevación moderadad de transaminasas. Los análisis de farmacodinámica demostraron la inhibición de la vía de mTOR con sirolimus a la dosis de 5 mg al día y los resultados de PK no encontraron que la concentración de sirolimus influyera en la eliminación de la gemcitabina. Los hallazgos de los estudios *in vitro* e *in vivo* sugieren que la vía de mTOR se hiperactiva con la administración de gemcitabina y que esa activación se revierte con sirolimus. El crecimiento tumoral de los xenografts de leiomiosarcoma fue espectacularmente inhibido por el tratamiento.

Conclusiones: La dosis recomendada fue sirolimus 5 mg al día más gemcitabina 800 mg/m2. Además, se observó actividad antitumoral en los modelos preclínicos de sarcoma, así como inhibición de la vía de mTOR.

9.4 Discusión

Nuestros resultados sugieren que la combinación de terapias dirigidas y drogas citotóxicas es una estrategia terapéutica segura y activa en sarcomas. Nuestros estudios preclínicos demuestran que combinar compuestos anti angiogénicos como el sorafenib con agentes citotóxicos como la ifosfamida tiene mayor actividad que cada uno de los fármacos por separado. Con la combinación del inhibidor de mTOR sirolimus y el agente citotóxico clásico gemcitabina, obtuvimos similares resultados en modelos *in vitro* e *in vivo* de sarcoma. Además, encontramos que la vía de mTOR se activa como resultado de la administración de gemcitabina, probablemente como mecanismo de resistencia tumoral a la quimioterapia, y que este efecto se revierte con sirolimus. En la clínica, demostramos que la combinación de ifosfamida más sorafenib

y la combinación de gemcitabina más sirolimus son regímenes factibles y seguros, permitiendo la administración de dosis activas de cada fármaco. También observamos signos precoces de actividad clínica.

A diferencia de otros tumores (229), a fecha de hoy no hay tratamientos aprobados con combinaciones de terapias dirigidas y drogas citotóxicas en sarcomas. Nuestros dos estudios demuestran que las combinaciones evaluadas tienen una toxicidad aceptable. Además, con las limitaciones de un estudio fase I para evaluar la eficacia clínica, la combinación de sorafenib más ifosfamida cumple con los criterios de supervivencia libre de progresión (SLP) propuestos por la Organización Europea para la Investigación y Tratamiento del Cáncer (EORTC) para el desarrollo de fármacos en sarcomas (211). Es más, la observación en los análisis de PK que el metabolito activo de la ifosfamida 4-hidroxi-ifosfamida aumenta con la administración concomitante de sorafenib, junto con los resultados aditivos observados *in vitro*, hacen esta combinación muy atractiva para seguir investigando (204). De hecho, en al menos dos ensayos clínicos fase II se ha evaluado esta estrategia terapéutica en sarcomas. Uno de ellos, realizado por nuestro grupo, es la continuación del estudio que aquí se presenta. En ese ensayo fase II, 35 pacientes con sarcoma avanzado que habían recibido doxorrubicina previa fueron tratados con la combinación de sorafenib e ifosfamida con la dosis y esquema recomendados en nuestro estudio fase I. El objetivo primario fue la tasa de SLP a los 3 meses, que fue del 67%. La mediana de SLP y de SG fue de 4,8 meses y 16,2 meses, respectivamente. En total, 17% de los pacientes obtuvieron respuesta parcial (RP). Estos resultados son superiores a los esperables con ifosfamida

en monoterapia, reforzando el interés de nuestro tratamiento de combinación (212). El otro estudio con sorafenib e ifosfamida realizado en sarcomas, específicamente en situación de neoadyuvancia, ha completado el reclutamiento pero los resultados aún no han sido comunicados (NCT00880542).

La estrategia terapéutica evaluada en nuestro segundo artículo, sirolimus más gemcitabina, también ha demostrado que merece ser investigada en mayor profundidad (213). En vista del favorable perfil de toxicidad observado en el estudio fase I, nuestro grupo realizó un ensayo clínico fase II en pacientes con sarcomas de partes blandas avanzados. Veintiocho pacientes que habían recibido varias líneas de tratamiento previas fueron incluidos y recibieron nuestra propuesta de combinación de sirolimus y gemcitabina. Los resultados preliminares fueron presentados en la reunión anual de 2014 de la Sociedad Americana de Oncología Clínica (ASCO) y mostraron que las tasas de SLP a 3 y 6 meses fueron 44% y 20%, respectivamente, cumpliendo el objetivo primario del estudio según las recomendaciones de la EORTC (211). La mediana de SLP fue 1,85 meses y la mediana de SG fue 9,1 meses (220). Estos resultados de eficacia pueden haber sido negativamente influidos por la población heterogénea y ampliamente pretratada que fue incluida en el estudio. El manuscrito con los resultados definitivos está siendo elaborado por nuestro grupo. Por otro lado, el Grupo Español de Investigación en Sarcomas (GEIS) ha evaluado esta combinación también en osteosarcoma, el sarcoma óseo más frecuente. En un estudio fase II, 36 pacientes con osteosarcoma avanzado recibieron la combinación de sirolimus y gemcitabina en segunda línea de tratamiento a la dosis y esquema recomendados en

nuestro estudio. Hubo 2 RP (6%), 13 enfermedades estables (39%) y 18 progresiones de enfermedad (54%) como mejores respuestas. La tasa de SLP a 4 meses, el principal objetivo del estudio, fue 44% y a 6 meses fue 28%. Las medianas de SLP y SG fueron 2,3 meses y 11,2 meses, respectivamente. Como en nuestro estudio, el perfil de toxicidad fue aceptable y el ensayo fue considerado positivo de acuerdo con los criterios de tasa de SLP definidos por los autores, lo que también refuerza nuestra estrategia combinatoria (221).

Estos resultados clínicos tan alentadores están respaldados por nuestras observaciones preclínicas. *In vitro*, encontramos que la administración secuencial de gemcitabina seguida de sirolimus se traduce en una mayor tasa de muerte celular y activación de la apoptosis comparada con cada una de las drogas en monoterapia y con la administración concomitante de ambos fármacos. *In vivo*, la misma combinación secuencial consiguió una inhibición muy importante del crecimiento tumoral, consiguiendo mucha mejor actividad anti tumoral comparado con cada droga en monoterapia. Además, encontramos tanto *in vitro* como *in vivo* el sorprendente hallazgo que la vía de mTOR se activa como resultado del tratamiento con gemcitabina, lo que puede representar un mecanismo de resistencia celular a la agresión del agente citotóxico. La activación de la vía de mTOR se inhibe cuando se añade sirolimus. Esta novedosa observación supone un mecanismo de resistencia a la quimioterapia en sarcomas potencialmente accionable que nunca se había reportado antes, lo que merece ser investigado en profundidad.

Ambos regímenes combinatorios aquí evaluados son estrategias terapéuticas prometedoras pero también tienen sus limitaciones. Probablemente, la más importante sea la ausencia de biomarcadores que guíen la selección de los pacientes que se beneficien de estos tratamientos. La identificación de biomarcadores predictivos es una cuestión no resuelta en sarcomas. Aparte del GIST, en el que la activación constitutiva del receptor KIT por mutaciones (222) es un factor predictivo de respuesta a inhibidores de KIT bien establecido (223), la caracterización molecular de los sarcomas aún no es relevante en la toma de decisiones de tratamiento. Es sabido que diferentes subtipos histológicos son más sensibles a responder a ciertas drogas que otros (18). Sin embargo, las características moleculares que subyacen detrás de esa sensibilidad especial a algunos tratamientos dependiendo de la histología aún no se conocen. Grandes esfuerzos en este campo tienen que realizarse en los próximos años para mejorar la supervivencia de los pacientes con sarcomas. Además, dado que la actividad de nuestros regímenes combinatorios ha sido confirmada en estudios clínicos fase II con una sola rama de tratamiento, el siguiente paso debería ser la comparación con otras terapias en estudios aleatorizados para determinar más exactamente su papel en el tratamiento de los sarcomas.

En resumen, en un grupo de tumores con tanta escasez de opciones de tratamiento como los sarcomas, cualquier aproximación terapéutica con una sólida base preclínica y unos prometedores signos de actividad como la combinación de sorafenib más ifosfamida y de siolimus más gemcitabina deberían ser exploradas exhaustivamente. Nuestros regímenes combinatorios propuestos, junto con esquemas secuenciales o

agentes de inmunoterapia, pueden ayudar en conseguir el objetivo final de mejorar el mal pronóstico de los pacientes afectos de sarcomas sin disminuir su calidad de vida. Ésta es una necesidad urgente que esperemos sea satisfecha en los próximos años.

9.5 Conclusiones

- En modelos *in vitro* de leiomiosarcoma y de sarcoma sinovial, la combinación de sorafenib e ifosfamida mostró mayor actividad que cada fármaco por separado, observándose un efecto aditivo.

- En la clínica, la combinación de sorafenib e ifosfamida es un tratamiento realizable y seguro, permitiendo la administración de dosis activas de ambos agentes y sin sinergismo en el perfil de toxicidad.

- Los análisis de PK no mostraron un descenso en los niveles plasmáticos de sorafenib ni de ifosfamida con la administración concomitante. La concentración del metabolito activo de la ifosfamida 4-hidroxi-ifosfamida aumenta con el sorafenib, sugiriendo que sorafenib puede aumentar los efectos de la ifosfamida sin incrementar la toxicidad.

- En nuestro estudio fase I con la combinación de sorafenib e ifosfamida se observaron signos precoces de actividad clínica en pacientes con sarcoma avanzado ampliamente

pre tratados. Estos hallazgos han sido confirmados en un ensayo clínico fase II con el mismo régimen terapéutico realizado por nuestro grupo.

- Tanto *in vitro* como *in vivo*, la administración de gemcitabina seguida de sirolimus consiguió una mayor actividad antitumoral que cada una de las drogas administradas en monoterapia o de forma concomitante en modelos de leiomiosarcoma y de sarcoma sinovial.

- En modelos preclínicos *in vitro* e *in vivo* de leiomiosarcoma y de sarcoma sinovial, la vía de señalización de mTOR es activada como resultado del tratamiento con gemcitabina lo que puede suponer un mecanismo de resistencia a la quimioterapia. Esta activación es revertida con la adición de sirolimus. Esto supone un nuevo hallazgo en sarcomas y un mecanismo de resistencia a la quimioterapia potencialmente accionable.

- En nuestro modelo de xenoinjerto de leiomiosarcoma, el crecimiento tumoral fue intensamente inhibido con la misma administración secuencial de sirolimus y gemcitabina, consiguiendo mucha mejor actividad anti tumoral que con cada droga en monoterapia.

- En la clínica, la combinación de sirolimus y gemcitabina es un tratamiento realizable y seguro, permitiendo la administración de dosis activas de ambos agentes y sin

sinergismo en el perfil de toxicidad. Los análisis de farmacodinamia demostraron inhibición de la vía de señalización de mTOR a la dosis recomendada y los análisis de PK no encontraron efectos de la concentración de sirolimus en la eliminación de gemcitabina.

- En un estudio fase II realizado por nuestro grupo con la combinación de sirolimus y gemcitabina utilizando la dosis recomendada por nuestro ensayo fase I se encontró actividad clínica en pacientes con sarcoma avanzado que habían sido ampliamente pretratados.

- Las combinaciones de sorafenib más ifosfamida y de sirolimus más gemcitabina son regímenes realizables y seguros con actividad clínica en sarcomas. Más investigación con estos tratamientos es necesaria.

10. References

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