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Abstract :

Anti-tumour therapies based on the use PARAs (pro-apoptotic receptor agonists), including TRAIL (TNF-Related Apoptosis inducing Ligand) or monoclonal antibodies targeting TRAIL-R1 or TRAIL-R2, have been disappointing so far, despite clear evidence of clinical activity and lack of adverse events for the vast majority of these compounds, whether combined or not with conventional or targeted anti-cancer therapies. This brief review aims at discussing the possible reasons for the lack of apparent success of these therapeutic approaches and at providing hints in order to rationally design optimal protocols based on our current understanding of TRAIL signalling regulation or resistance for future clinical trials.

Keywords : TNF-receptor superfamily; TRAIL; Fas; Therapy; Apoptosis; Tumor targeting; Resistance

Abbreviations; **DISC:** Death-Inducing Signaling Complex; **PARAs:** pro-apoptotic receptor agonists; **TNF:** Tumour Necrosis Factor; **TRAIL:** TNF-Related Apoptosis-Inducing Ligand

Introduction :

Apoptosis is crucial for tissue homeostasis and normal development. Its dysregulation often occurs in cancers and has been implicated in many events relevant to the pathogenesis, progression or chemoresistance of tumours. Restoring sensitivity to and exploiting the induction of apoptosis as a mean to eradicate cancer cells has thus been considered attractive as a potential cure for patients for the last two decades (Kerr *et al.*, 1994). The apoptotic machinery can be engaged by two main signalling pathways: the mitochondrial-dependent intrinsic pathway that is mostly engaged by conventional chemotherapeutic drugs (Fulda *et al.*, 2006), and the extrinsic pathway that can be triggered by ligands of the TNF or Toll superfamily, such as TRAIL, Fas, TNF- α , or dsRNA (Ashkenazi *et al.*, 1998; Estornes *et al.*, 2012; Merino *et al.*, 2007; Weber *et al.*, 2010).

Unlike most chemotherapeutic drugs (Fridman *et al.*, 2003), ligands of the TNF family engage apoptosis in a p53-independent manner and have thus been considered as an alternative to conventional chemo- or radiotherapy owing to the fact that p53 mutations are often found in tumours, ranging from 10 to 80%, depending on the type (Greenblatt *et al.*, 1994; Nigro *et al.*, 1989). Binding of these ligands to their cognate receptors induces receptor aggregation and the formation of a macromolecular complex, coined DISC for Death-Inducing Signalling Complex, which depending on the cellular context may lead to apoptosis (Figure 1). The DISC forms due to homotypic interactions by means of the death domain (DD) and death effector domains (DEDs) of adaptor proteins such as FADD or TRADD and the initiator caspases, procaspase-8 and -10. DISC assembly ensures procaspase-8/10 oligomerization and activation, leading to subsequent cleavage and release of the active initiator caspase. While activation of caspase-8/10 within the DISC occurs at the plasma membrane upon Fas ligand and TRAIL stimulation, recruitment and activation of initiator caspases upon TNF-R1 engagement takes place in a cytosolic complex generated sequentially from TNF-R1 DISC (Figure 1), coined complex II, arising from the primary membrane-bound complex that contains TRADD, TRAF-2, RIP, cIAPs and IKKs but lacks FADD and caspase-8 (Micheau *et al.*, 2003). Recently it has been demonstrated that formation of complex II is negatively regulated through linear ubiquitination by LUBAC, a complex composed of HOIL-1, HOIP and sharpin, also recruited within complex I (Haas *et al.*, 2009). Independently of whether initiator caspases are activated at the membrane or within the cytosolic compartment, their activation triggers a proteolytic cascade leading to apoptosis either directly or through an amplification loop involving mitochondria (Figure 1).

TNF- α and Fas ligand:

Clinical studies aiming at evaluating the anti-tumoral efficacy of TNF family members were initiated three decades ago with recombinant human TNF- α (for a recent review see (Roberts *et al.*, 2011)). Unfortunately, systemic TNF- α treatments induced severe adverse events including hepatotoxicity and hypotension. Clinical activity was rarely obtained, consistent with TNF- α 's inability to trigger apoptosis or cell death in most tumour cells, unless the initial NF- κ B pathway that is triggered by the membrane-bound complex fails to be activated (Figure 1 and (Micheau *et al.*, 2001)). By contrast TNF- α very efficiently triggers apoptosis of endothelial cells (Ruegg *et al.*, 1998) and improves drug uptake by the tumour (van der Veen *et al.*, 2000). These properties are now successfully used in clinical trials to treat limb-threatening soft tissue sarcomas (Deroose *et al.*, 2012) or in-transit melanoma metastases (Rossi *et al.*, 2010), in the setting of hyperthermic isolated limb perfusion with low-dose of TNF- and melphalan.

Like TNF- α , Fas ligand is highly cytotoxic towards primary hepatocytes and other non-transformed cells and has been shown to induce fulminant liver injuries in rodents (Costelli *et al.*, 2003; Ogasawara *et al.*, 1993). However, contrary to TNF- α , Fas ligand is a potent activator of apoptosis in tumour cells. Importantly, despite the fact that most ligands of the TNF family are naturally found as trimers, engagement of the apoptotic machinery by Fas can only be triggered by the use of hexameric ligands (Schneider *et al.*, 1998) or by antibody-mediated trimeric ligand cross-linking (Berg *et al.*, 2007). In line with these findings, a novel Fas ligand preparation coined APO010 has been generated (Holler *et al.*, 2003) and is now being evaluated for its antitumoral properties (Figure 1 and 2A). This hexameric ligand, which has been obtained by fusing the

collagen domain of adiponectin to Fas ligand extracellular domain (Holler *et al.*, 2003), appears to be safe *in vivo* (Etter *et al.*, 2007). APO010 was shown to be an effective anticancer agent in *in vitro* and *in vivo* preclinical studies (Eisele *et al.*, 2011; Verbrugge *et al.*, 2010; Verbrugge *et al.*, 2009). Locoregional administration of APO010 prolonged the survival of mice bearing peritoneal tumour xenografts (Etter *et al.*, 2007). This preparation of Fas ligand is now being evaluated in a phase I dose-escalation study (NCT00437736), to determine its safety and tolerability after intravenous bolus injection in patients with solid tumours.

TRAIL and derivatives

TRAIL recombinant proteins :

Within the TNF superfamily, TRAIL is the ligand that has attracted the most interest in oncology owing to its ability to induce apoptosis in transformed cells but not in normal cells and to its lack of toxicity in animal models (Ashkenazi *et al.*, 1999; Drosopoulos *et al.*, 2005; Nesterov *et al.*, 2004; Nieminen *et al.*, 2007; Walczak *et al.*, 1999). The first human recombinant TRAIL preparation, evaluated in clinical studies, was generated by Genentech (Figure 2A). This version of TRAIL, termed APO2L/TRAIL.0 and later on dulanermin, was obtained from bacteria as a non-tagged native recombinant protein encoding amino acid residues 114-281 of human TRAIL (Lawrence *et al.*, 2001). Similar to TNF or Fas ligand, TRAIL is naturally found as a homotrimeric ligand (Hymowitz *et al.*, 1999). However, TRAIL harbours a unique feature, a central zinc atom that binds to cysteine side chains from each trimer subunit and which displays important regulatory function. This coordinated zinc atom has been reported to play a crucial role not only for protein stability and solubility but also for TRAIL's biological activity (Bodmer *et al.*, 2000; Hymowitz *et al.*, 2000). Native TRAIL preparations were found superior to poly-histidine-tagged human TRAIL preparations in triggering cancer cell apoptosis, and importantly, contrary to his-tagged TRAIL, native TRAIL is poorly cytotoxic to hepatocytes (Jo *et al.*, 2000). As reported from clinical studies (Herbst *et al.*, 2010a; Herbst *et al.*, 2006; Ling *et al.*, 2006), dulanermin can be safely administered intravenously in patients and is well tolerated up to 30 mg/kg daily for 5 days every 3 weeks (Table 1). Common reported adverse events include fatigue, nausea, vomiting, fever, anemia and constipation for 18 to 38% of the patients. Severe adverse events were reported in two patients. Most adverse events were related to disease progression or concomitant illness. Dulanermin's plasmatic concentrations were found to be compatible with most preclinical studies, ranging from 5 µg/ml to 220 µg/ml at 0.5 and 30 mg/kg administration doses, respectively. Pharmacokinetic parameters are not influenced by gender, race or enzymatic activities such as alkaline phosphatase or aspartate aminotransferase (Xin *et al.*, 2008). The half-life of dulanermin, however, is relatively short and doesn't exceed 1 hour, with no apparent accumulation in the serum (Herbst *et al.*, 2010a). Despite clear evidence of biological activity as determined by the increase in serum caspase-3/7 activation levels in more than 50 % of the patients treated with dulanermin (Pan *et al.*, 2007; Pan *et al.*, 2011), clinical antitumor activity was only evidenced in two patients with chondrosarcomas, who experienced partial response to dulanermin. These patients have been treated with dulanermin for 2 and more than 3 years, at the time of the submission of the manuscript (Herbst *et al.*, 2010a). Because preclinical studies largely demonstrate that a chemotherapy regimen can overcome resistance to TRAIL-induced apoptosis (Ashkenazi *et al.*, 2008; El Fajoui *et al.*, 2011; Ganten *et al.*, 2004; Gliniak *et al.*, 1999; Jacquemin *et al.*, 2012; Jacquemin *et al.*, 2010; Keane *et al.*, 1999; Lacour *et al.*, 2003; Merino *et al.*, 2007; Morizot *et al.*, 2011; Singh *et al.*, 2003), with minimal toxicities (Evdokiou *et al.*, 2002; Ganten *et al.*, 2006; Ravi *et al.*, 2004), several clinical studies have been designed to assess TRAIL anti-tumoral potential in combination with conventional or targeted anti-cancer therapies (Table 2). Dulanermin has been combined with rituximab in low grade non-hodgkin's lymphomas (Belada *et al.*, 2010; Yee *et al.*, 2007), to carboplatin, paclitaxel and bevacizumab in advanced tumours and first line advanced stage III/IV non small cell lung carcinomas (Blackhall *et al.*, 2010; Soria *et al.*, 2011; Soria *et al.*, 2010), to FOLFOX and bevacizumab in untreated, advanced or recurrent metastatic colorectal cancers (Kozloff *et al.*, 2012), to an anti-IGR1 in advanced refractory solid tumours (NCT00819169), to irinotecan and cetuximab or FOLFIRI in metastatic colorectal carcinomas (Yee *et al.*, 2009), and to camptosar and erbitux or FOLFIRI plus or minus bevacizumab in previously untreated metastatic colorectal carcinomas (NCT00671372 and (Kasubhai *et al.*, 2012)). Overall, with the exception of the anti-IGFR1, whose clinical trial was terminated for a reason that was not reported, combining dulanermin with these chemotherapeutic regimens appeared to be rather well tolerated by patients.

Incidences of adverse events (AE) were relatively similar across the different treatments arms and most fatal AE were related to disease progression. However, combining dulanermin with these compounds didn't improve objective response rates, neither overall patient survival. Reasons for this lack of efficacy are not clear for the moment, although some concerns may be pointed out regarding these combinations and the design of these studies which most of the time are poorly concordant with preclinical studies. One clinical study is still in progress to evaluate the efficacy of dulanermin combined with camptosar and erbitux or FOLFIRI in association or not with bevacizumab in previously untreated metastatic colorectal carcinomas (NCT00671372).

More recently, another TRAIL preparation, consisting of the fusion of the human TRAIL amino acid residues 135-280 fused to residues 122-135 of (Figure 2A), also coined CPT or circularly permuted TRAIL, has entered clinical trials. Early preclinical studies evaluating CPT demonstrated its potential anti-tumoral properties alone and in association with chemotherapy *in vitro* and *in vivo* on several tumour types (Fang *et al.*, 2005; Tang *et al.*, 2008; Tang *et al.*, 2005; Wang *et al.*, 2008; Zhang *et al.*, 2006). A phase Ib dose escalation study (Chen *et al.*, 2012b) and a phase II multi-center open label single arm study (Chen *et al.*, 2012c) have been performed on relapsed or refractory multiple myeloma patients. CPT was either given intravenously for 5 consecutive days each 21 days for 4 cycles with increasing concentrations ranging from 5 to 15 mg/kg per day or administered at 2.5 mg/kg per day for 14 consecutive days of each 21-day cycle for 2 cycles. In the phase Ib study, CPT was found to be well tolerated up to 15 mg/kg with no dose limiting toxicity and limited adverse events, including fever, leucopenia, elevated AST, fatigue and vomiting. Similar to dulanermin, CPT's half-life was estimated to 1 hour. Of the 21 patients treated with CPT one achieved complete response and 4 underwent partial responses. In the phase II study, a similar overall response rate of 33% was found for the 27 patients enrolled, with one patient experiencing near complete response and 8 patients exhibiting partial responses. Of note, one patient experienced a severe adverse event, the occurrence of which was attributed to CPT-mediated liver injury. Interestingly, a third clinical study has been presented at the American Society of Haematology, associating CPT and thalidomide (Chen *et al.*, 2012a). This multiple-center, open-label, single arm phase II study enrolled 43 second line relapsed or refractory multiple myeloma patients resistant to thalidomide. Patients received thalidomide daily and CPT at 5, 8, or 10 mg/kg per day during the first 5 days of a 21-day cycle, up to 6 cycles or until disease progression or intolerant adverse events. Amongst the 41 patients that could be evaluated, 2 achieved complete response, 3 near complete response and 4 underwent partial responses. Of the 3 groups of patients, best responses were obtained at the highest CPT doses 10 mg/kg. No major adverse event was attributed to CPT. Overall, the authors of this study concluded the combination associating thalidomide and CPT was not only well tolerated, but displayed superior anti-tumoral properties and clinical activities as CPT alone.

Anti-TRAIL-R1 agonistic monoclonal antibody

In addition to TRAIL preparations, alternative therapeutic strategies based on agonistic monoclonal antibodies (MoAb), that specifically target TRAIL receptors TRAIL-R1 or TRAIL-R2 have been considered, to avoid TRAIL resistance induced by TRAIL binding to the two antagonistic receptors TRAIL-R3 and TRAIL-R4 (Davidovich *et al.*, 2004; Marsters *et al.*, 1997; Meng *et al.*, 2000; Merino *et al.*, 2006; Riccioni *et al.*, 2005; Toscano *et al.*, 2008). So far, only one anti-TRAIL-R1 agonistic MoAb has been evaluated in clinical studies. Mapatumumab, also coined TRM1 or HGS-ETR1 has been assessed in phase I and II clinical trials (Table 1), in advanced solid tumours (Tolcher *et al.*, 2007), advanced hepatocellular carcinomas (Sun *et al.*, 2011), refractory and relapsed non-hodgkin's lymphomas (Younes *et al.*, 2010), refractory colorectal carcinomas (Trarbach *et al.*, 2010) and relapsed or recurrent stage III and IV non-small cell lung carcinomas (NSCLC) (Greco *et al.*, 2008). From these studies, it was found that mapatumumab is safe and well tolerated up to 20 mg/kg per day (Hotte *et al.*, 2008; Tolcher *et al.*, 2007; Trarbach *et al.*, 2010; Younes *et al.*, 2010). Mapatumumab-induced adverse events were mostly grade 1 and 2, including fatigue, hypotension and nausea. However in a phase I study two patients at the highest dose level experienced dose limiting effects consisting of grade 3 transaminase and bilirubin elevations, probably associated with mapatumumab (Tolcher *et al.*, 2007). Plasmatic concentrations were found to be largely compatible with preclinical studies ranging from 0.27 to 400 µg/ml at the corresponding 0.01 to 20 mg/kg doses. From these studies, however, mapatumumab only showed clinical activity in patients with refractory or relapsed non-hodgkin's lymphomas and in particular in patients with follicular lymphomas. Out of 15 patients harbouring follicular lymphomas, two experienced a complete response and one a partial response (Younes *et al.*, 2010). Clinical studies to evaluate the anti-

tumoral efficacy of mapatumumab combined with chemotherapy have also been performed and some are still ongoing (Table 3). Of these, a phase I study associating mapatumumab with gemcitabine and cisplatin in patients with advanced solid tumours (Mom *et al.*, 2009), reported that this anti-TRAIL-R1 MoAb could exhibit clinical activity. Likewise 26 out of 37 patients receiving mapatumumab experienced decreased tumour lesions, 12 patients achieved partial responses and 25 of them experienced stable disease. In another phase I study, the association of mapatumumab with paclitaxel and carboplatin (Leong *et al.*, 2009), was also suggested to exhibit clinical activity with 5 patients achieving confirmed partial responses and 12 patients with stable disease. From these two studies it was found that these combinations are rather safe and that the pharmacokinetic parameters of each of these compounds, alone, were apparently not affected by the combination (Chow *et al.*, 2006). However more recently, preliminary results of a phase II randomized study, performed on stage III and IV NSCLC patients receiving carboplatin and paclitaxel combined or not to mapatumumab as a first line therapy, clearly suggest that mapatumumab did not improve the response rate nor the progression free survival of NSCLC patients treated with carboplatin and paclitaxel (Von Pawel *et al.*, 2010). Moreover, a phase II randomized study in relapsed or refractory multiple myeloma demonstrated that mapatumumab combined with bortezomib was not better than bortezomib alone (Belch *et al.*, 2010). Another platinum-based combination is currently recruiting patients in a phase Ib/II to assess the efficacy of first line therapy associating mapatumumab, cisplatin and radiotherapy on cervical cancers (NCT01088347). In spite of these rather disappointing results, the use of TRAIL-R1 monoclonal antibodies may still be of interest for some tumour types which respond to TRAIL-R1, such as lymphoid malignancies (Younes *et al.*, 2010), or melanomas (Kurbanov *et al.*, 2005).

Anti-TRAIL-R2 agonistic monoclonal antibodies

For an unknown reason, more agonistic monoclonal antibodies specifically targeting TRAIL-R2 have been generated than those for TRAIL-R1. So far, 5 anti-TRAIL-R2 MoAb have been assessed in clinical trials, namely conatumumab (or AMG655), drozitumumab (also coined Apomab or PRO95780), LBY135, lexatumumab (or HGS-TR2) and tigatuzumab (also called CS-1008 or TRA-8). Like TRAIL and mapatumumab, these agonistic anti-TRAIL-R2 antibodies have entered the clinical studies alone or in association with chemotherapeutic drugs. With the exception of drozitumumab that has been suspected to induce possible hepatic adverse events in 4 patients out of 50, most TRAIL-R2 agonistic antibodies were found to be well tolerated by patients at doses ranging from 8 to 20 mg/kg per day (Table 1). Their half-life was found to range from 8 to 19 days and clinical efficacies were reported for a large range of tumours.

Conatumumab

Conatumumab was suggested to exhibit anti-tumoral activity in advanced solid tumours (Doi *et al.*, 2011; Herbst *et al.*, 2010b; LoRusso *et al.*, 2007). Combined studies report divergent results depending on the combination (Table 4). Some clinical activities of conatumumab were reported in association with ganitumab (an anti-IGFR1 monoclonal antibody) in advanced refractory solid tumours (Chawla *et al.*, 2010), with FOLFIRI and ganitumab in second line treatment KRAS mutant metastatic colorectal cancers (Cohn *et al.*, 2012) and with gemcitabine associated or not with ganitumab in metastatic pancreatic cancers (Kindler *et al.*, 2010). However, in unresectable soft tissue sarcomas, conatumumab was found to decrease the overall response rate of doxorubicin (Demetri *et al.*, 2012). Four other clinical studies have been performed or are ongoing. Amongst these it should be noted that one of these, evaluating conatumumab in association with bortezomib or vorinostat in relapsed or refractory lymphomas (NCT00791011), has been suspended and two others evaluating conatumumab in association with panitumumab (an anti-EGFR monoclonal antibody) in metastatic colorectal cancers (NCT00630786), or paclitaxel and carboplatin in first-line advanced NSCLC (NCT00534027), have been completed, but the results have not been divulged. The last ongoing study is not recruiting patients and aims at evaluating conatumumab efficacy in association with FOLFOX6 and bevacizumab in first-line metastatic colorectal cancer patients (NCT00625651).

Drozitumab

So far, no objective response has been attributed to the administration of drozitumab in patients with advanced solid tumours (Table 1). Only minor responses have been described in 2 patients with colorectal and granulosa cell ovarian cancers and one patient with a chondrosarcoma (Camidge *et al.*, 2010). However, a terminated phase II study in patients with chondrosarcoma, suggests a lack of efficacy of drozitumab for this population (NCT00543712). A second phase II study in NSCLC patients has been terminated, but the results are still awaited (NCT00480831). With combined studies, results have also been disappointing. In association with rituximab in patients with relapsed non-hodgkin's lymphoma (Wittebol *et al.*, 2010) or with paclitaxel, carboplatin and bevacizumab in previously untreated stage IIIb and IV NSCLC patients (Karapetis *et al.*, 2010), drozitumab failed to demonstrate clinical activity. In two other studies associating drozitumab with cetuximab and irinotecan or FOLFIRI and bevacizumab (Baron *et al.*, 2011), or FOLFOX and bevacizumab in first line metastatic colorectal cancer patients (Rocha Lima *et al.*, 2012; Rocha Lima *et al.*, 2011) some antitumor activity has been described (Table 4), yet drozitumab development appears to have been placed on hold and currently there are currently no additional clinical trials ongoing.

LBY135

LBY135 is another anti-TRAIL-R2 agonistic antibody that has been assessed in clinical trials (Table 1 and 4). LBY135 alone in solid advanced tumours was shown to be safe and well tolerated up to 20 mg/kg (Sharma *et al.*, 2008). Some clinical activities were reported. One patient with sarcoma experienced a minor response and 2 patients with NSCLC or prostate cancer had a decrease in tumour markers of 50% and 40% respectively. Combined with capecitabine, a precursor of 5-FU, LBY135 induced 2 partial responses in ovarian and colorectal cancer patients and 60-73% tumour mass reduction in 4 patients with ovarian, colorectal and pancreatic cancers.

Lexatumumab

Despite the large number of preclinical studies demonstrating its anti-tumoral activity, lexatumumab has poorly been assessed in the clinic. Alone, lexatumumab was shown to be safe and well tolerated up to 10 mg/kg per day and its half-life has been estimated to 2-18 days, depending on the study. In advanced solid tumours (Plummer *et al.*, 2007), lymphomas (Wakelee *et al.*, 2010) and pediatric solid tumours (Merchant *et al.*, 2012), lexatumumab showed signs of clinical activity (Table 1). The most striking activity was achieved in a teenager with progressive, unresectable chest wall/lung osteosarcoma, who was treated for 2 years with lexatumumab and experienced resolution of symptoms, ossification of her lesion, and loss of PET activity. She remained symptom free with stable imaging for more than 1 year after cessation of therapy. Strikingly, combined studies associating lexatumumab and chemotherapy regimens have been scarce, compared to other anti-TRAIL-R2 agonistic monoclonal antibodies (Table 4). Evidence for clinical activity have only been reported in a study combining lexatumumab with gemcitabine, pemetrexed, doxorubicin or FOLFIRI in advanced solid tumours (Sikic *et al.*, 2007). In this study it was found that the combination induced some tumour shrinkage and partial responses in the FOLFIRI and doxorubicin arm. The second trial combining INF γ in refractory pediatric solid tumours (NCT01445093) has been completed, but so far, results have not been published.

Tigatuzumab

The only dose-escalating phase I reported for tigatuzumab enrolled 16 relapsed or refractory solid tumours or lymphoma patients (Forero-Torres *et al.*, 2010). Tigatuzumab was well tolerated up to 8 mg/kg per day with no dose limiting toxicity and a half-life estimated to 6-10 days. Tigatuzumab showed signs of clinical activity with 7 patients experiencing stable disease, despite the fact that most of these patients have been heavily pretreated with chemotherapy regimens prior to enrolment. Remarkably, one patient entering the trial with progressive

metastatic hepatocellular carcinoma, suffering from pain and having failed to respond to alkylating agents, topoisomerase and microtubule inhibitors, became pain free 6 weeks after the onset of the treatment and remained asymptomatic with stable disease for more than 26 months (Forero-Torres *et al.*, 2010). Clinical studies combining tigatuzumab and chemotherapy regimens have been conducted but results have not yet been communicated (Table 4). Tigatuzumab has been assessed combined with paclitaxel and carboplatin in metastatic or unresectable NSCLC (NCT00991796), with gemcitabine in untreated and unresectable pancreatic cancers (NCT00945191) and with irinotecan in metastatic colorectal cancer patients who failed first-line treatments with oxaliplatin (NCT00521404). Three other studies are ongoing. A phase I study combining FOLFIRI and tigatuzumab in patients with metastatic colorectal cancer who have failed first-line treatments that were not based on irinotecan (NCT01124630) and two phase II randomized studies combining sorafenib in advanced liver cancer patients (NCT01033240) or paclitaxel in metastatic triple negative breast cancers (NCT01307891). Results of these studies are eagerly awaited.

Study design and administration of TRAIL and derivatives: lessons from preclinical studies

With the exception of the newly designed recombinant TRAIL, CPT, clinical studies aiming at evaluating the efficacy of dulanermin or agonistic antibodies targeting TRAIL-R1 or TRAIL-R2, as single agents or combined with chemotherapy regimens, have been disappointing despite clear evidence of efficacy in a, so far, limited number of cases. Understanding how TRAIL signalling is regulated, in light of these results, appears more crucial than ever in order to design appropriate clinical trials aiming at evaluating the anti-tumoral properties of TRAIL preparations and TRAIL derivatives. The TRAIL system is probably the most complex system of the TNF superfamily owing to the ability of TRAIL to bind to five different receptors (Shirley *et al.*, 2011). With the exception of OPG (osteoprotegerin), which displays low affinity to TRAIL, the 4 other receptors, namely TRAIL-R1, TRAIL-R2, TRAIL-R3 and TRAIL-R4, bind TRAIL with high affinity. However, only TRAIL-R1 and TRAIL-R2 can engage apoptosis, owing to the presence within their intracellular domain of a particular motif, called the death domain through which components of the DISC are assembled (Ashkenazi *et al.*, 1998).

The first requirement to assess TRAIL-based anti-tumoral efficacy would certainly deserve, whenever possible, evaluation of TRAIL receptor expression levels in tumours biopsies. The lack of expression of these receptors for a given patient would obviously lead to resistance. While this might appear trivial, this is not an easy goal to achieve. Likewise, most immunohistochemical studies performed so far to evaluate TRAIL receptor expression in primary tumour tissues are poorly informative due to a lack of specific evaluation of membrane-bound expression levels of these receptors. Nonetheless, heterogeneous expression levels of TRAIL receptors have been found in a large variety of tumours (Cooper *et al.*, 2008; Ganten *et al.*, 2009; Granci *et al.*, 2008; Kuijlen *et al.*, 2006; Leithner *et al.*, 2009; Macher-Goeppinger *et al.*, 2009; McCarthy *et al.*, 2005; Min *et al.*, 2004; Pordzik *et al.*, 2011; Strater *et al.*, 2002; van Geelen *et al.*, 2006). However, their expression level is probably overestimated by the fact that most of these studies revealed mainly cytoplasmic expression. In line with this concern it has been demonstrated that membrane expression levels of TRAIL-R1 and TRAIL-R2 in acute myeloid leukemia did not exceed 50 and 12,5%, respectively (Riccioni *et al.*, 2005). In hepatocellular carcinomas (HCC), a particularly well-designed and informative study demonstrates that TRAIL-R1 and TRAIL-R2 expression were found restricted to the membrane in 30% and 15% of HCC primary tumours, respectively, but an additional fraction of these tumours, 36% and 3% expressed TRAIL-R1 and TRAIL-R2, respectively, both at the membrane and in the cytosol, (Kriegl *et al.*, 2010). As compared to surrounding non-tumourous cells, overall expression levels decreased in HCC with nearly 3% of the tumours lacking TRAIL-R1 and 46% lacking TRAIL-R2, and almost 30% of them expressing TRAIL-R1 and TRAIL-R2 only in the cytoplasm. From this study, it would be anticipated that almost 33 to 82% of these primary tumours would have lost or exhibit non-functional TRAIL-R1 and TRAIL-R2. Furthermore, it is becoming clear that selective engagement of either TRAIL-R1 or TRAIL-R2 can occur in a variety of tumours. Likewise, it has been demonstrated that chronic lymphocytic leukaemia and mantle cell lymphoma (MacFarlane *et al.*, 2005a; MacFarlane *et al.*, 2005b; Naton *et al.*, 2007) or pancreatic carcinomas (Lemke *et al.*, 2010; Stadel *et al.*, 2010) signal to apoptosis almost exclusively through TRAIL-R1, regardless of TRAIL-R2 membrane expression levels, whereas apoptosis in human glioma (Nagane *et al.*, 2010) or p53 wt myeloma cells (Surget *et al.*, 2012) mainly involves TRAIL-R2. Thus, since we are still unable to predict which TRAIL receptors are functional for a given tumour, these findings are likely to provide a satisfactory answer to the limited efficacy of some of the PARAs used alone in clinical trial, and in particular to the poor efficacy of mapatumumab. Moreover, the efficacy of dulanermin or CPT may specifically be compromised in tumours cells expressing TRAIL-R3 or TRAIL-R4 (Figure 2). We and others have shown that these antagonistic receptors negatively regulate apoptosis induced by recombinant TRAIL preparations (Bouralexis *et al.*, 2003; Jacquemin *et al.*, 2012; Meng *et al.*, 2000; Merino *et al.*, 2006; Sanlioglu *et al.*, 2007) while sparing that induced by selective anti-TRAIL-R1 or -TRAIL-R2 agonistic antibodies or peptidomimetics targeting TRAIL-R2 (Pavet *et al.*, 2010). However, like TRAIL-R1 and TRAIL-R2, immunohistochemical studies describing high TRAIL-R3 and TRAIL-R4 expression levels in primary tumours mostly document cytoplasm expression but rarely membrane expression levels. Yet, the study performed by Riccioni *et al.*, assessing TRAIL receptor expression levels at the membrane in acute myeloid leukemia demonstrates that TRAIL-R3 and TRAIL-R4 are highly expressed in more than 60% of cases (Riccioni *et al.*, 2005). Whether these receptors are also expressed at the cell surface in other types of tumours certainly remains to be determined more carefully since the latter, although not exclusively, are likely to compromise anti-tumoral therapies based on the use of recombinant TRAIL preparations.

In addition to their spontaneous expression by primary tumours, expression of these antagonistic receptors at the membrane is likely to be induced by some chemotherapy regimens, prior enrolment or during combined studies. We have recently demonstrated that whilst oxaliplatin is able to synergize with TRAIL in p53-deficient or mutated colorectal tumour cells (El Fajoui *et al.*, 2011), this compound triggers a p53-dependent up-regulation of TRAIL-R3 that impairs the benefit of the combination in wild type p53 colorectal tumour cells, without affecting expression levels of the other receptors (Toscano *et al.*, 2008). The reasons for the selective up-regulation of TRAIL-R3 by oxaliplatin in these cells remains unclear since it has been demonstrated that the promoters of all four TRAIL receptors contain functional p53 sites (Liu *et al.*, 2005; Liu *et al.*, 2004; Ruiz de Almodovar *et al.*, 2002; Takimoto *et al.*, 2000; Wu *et al.*, 1997). Nonetheless, enforced p53 activation using adenoviruses induces TRAIL-R4 expression and cross-resistance to chemotherapy-induced cell death (Liu *et al.*, 2005; Meng *et al.*, 2000). Likewise, ectopic expression of TRAIL-R4 in some tumour cells impairs apoptosis induced by chemotherapy (Lalaoui *et al.*, 2011). In agreement with these studies, it has been found that inactivation of TRAIL-R2 or mouse TRAIL-R expression conferred resistance to chemotherapy (Wang *et al.*, 2004) and radiotherapy (Finnberg *et al.*, 2005). It therefore cannot be excluded that some chemotherapy regimens may lead to TRAIL resistance or that selection of resistant tumours induced by TRAIL treatments may give rise to cross-resistance to chemotherapy through progressive acquisition of antagonistic receptor expression (Bouralexis *et al.*, 2003) or loss of agonistic TRAIL receptors (Finnberg *et al.*, 2005; Wang *et al.*, 2004).

TRAIL pro-apoptotic signalling pathway is regulated by three main checkpoints that can cooperate to inhibit full execution of the machinery (Figure 2D). The first checkpoint, at the membrane, is highly specific and tightly controlled by receptor expression levels, whether by loss of TRAIL-R1 or TRAIL-R2 expression, overexpression of the antagonistic receptors, TRAIL-R3 or TRAIL-R4 or by post-translational modifications through O-glycosylation. The second, less specific but nonetheless potent, checkpoint at the DISC level is mostly under the control of c-FLIP, the endogenous inhibitor of the initiator caspases-8 and -10, and is also involved in regulating apoptosis-induced by the other receptors of the TNF superfamily and TLR3. Last but not least, the third checkpoint, which is also involved in chemotherapy-induced cell death, occurs at the mitochondrial level.

As discussed above, one possible explanation for the lack of efficacy of these PARAs could reside in the poor expression level of TRAIL-R1 or TRAIL-R2 in some tumours (Duiker *et al.*, 2010; Ganten *et al.*, 2009; Kriegl *et al.*, 2010), and/or result from selective engagement of either TRAIL-R1 or TRAIL-R2 (MacFarlane *et al.*, 2005a; MacFarlane *et al.*, 2005b; Nagane *et al.*, 2010; Stadel *et al.*, 2010; Surget *et al.*, 2012) or regulation of the antagonistic receptors TRAIL-R3 or TRAIL-R4 and/or downstream inhibitors of TRAIL signalling by chemotherapeutic drugs (Bouralexis *et al.*, 2003; Davidovich *et al.*, 2004; Lalaoui *et al.*, 2011; Liu *et al.*, 2005; Morizot *et al.*, 2011; Nguyen *et al.*, 2006; Toscano *et al.*, 2008). TRAIL receptor expression in patients having received first line chemotherapy based on DNA-damaging drugs or in protocols associating TRAIL and DNA-damage compounds should thus be assessed carefully to evaluate the efficacy of these combinations in clinical studies.

Alternatively, lack of efficacy of some of these PARAs may be explained by their poor efficiency in triggering apoptosis. It has been demonstrated for example that some humanized anti-TRAIL receptor antibodies require cross-linking to achieve optimal activity in *in vitro* studies, and that most of them display lower proapoptotic activity as compared to recombinant TRAIL preparations (Chuntharapai *et al.*, 2001; Dobson *et al.*, 2009; Jin *et al.*, 2008; Kang *et al.*, 2011; Yada *et al.*, 2008; Zinonos *et al.*, 2009). In line with this hypothesis is the demonstration that the newly formulated TRAIL preparation CPT, which displays stronger pro-apoptotic activity than dulcanermin (Fang *et al.*, 2005), appeared to be more efficient than dulcanermin or monoclonal antibodies targeting TRAIL-R1 or TRAIL-R2 in early clinical trials, either alone or combined with chemotherapy. However, so far CPT has only been assessed in relapsed or refractory multiple myeloma in association or not with thalidomide, and additional clinical trials may be necessary to fully demonstrate that CPT preparations are superior to dulcanermin or anti-TRAIL receptor antibodies. Nonetheless, these results are encouraging and suggest that it may be possible to engineer TRAIL derivatives that could display higher anti-tumoral properties and that may be of interest for oncologists (Reis *et al.*, 2009; Szegezdi *et al.*, 2012; van der Sloot *et al.*, 2006). In addition, although it had been demonstrated that engagement of apoptosis and DISC

formation by dulcanermin requires TRAIL receptor O-glycosylation, and that these post-translational modifications have no impact on TRAIL binding to TRAIL-R2 (Wagner *et al.*, 2007), it remains unclear whether these modifications are required for monoclonal antibodies targeting TRAIL receptors to engage apoptosis and whether they might compromise binding of some of these antibodies to their target. Less specifically, poor clinical activities may also be explained by high expression levels of c-FLIP or anti-apoptotic proteins of the bcl-2 family, both of which are found to be highly expressed in a large number of tumours (Bagnoli *et al.*, 2009; Du *et al.*, 2009; Duiker *et al.*, 2010; Garcia *et al.*, 2002; McLornan *et al.*, 2013; Tolcher, 2005).

Notwithstanding the molecular mechanisms that are required to efficiently engage TRAIL-induced cell death machinery in primary tumour cells, inappropriate administration schemes most likely provide the best explanation for the poor efficacy of PARAs combined with chemotherapy regimens in clinical studies. It has been found in the past that chemotherapy and TRAIL, used simultaneously can afford restoration of apoptosis in a large number of TRAIL- or chemo-resistant tumour cells *in vitro* and *in vivo* (Cuello *et al.*, 2001; Fiveash *et al.*, 2008; Gliniak *et al.*, 1999; Jin *et al.*, 2004; Keane *et al.*, 1999; Lacour *et al.*, 2003; Ohtsuka *et al.*, 2003; Shankar *et al.*, 2004; Walczak *et al.*, 2000), but these synergies were shown to critically rely on mitochondrial activation (Nguyen *et al.*, 2006; von Haefen *et al.*, 2004). Unfortunately, a large proportion of tumours, and not only haematological malignancies, spontaneously overexpress Bcl-2 anti-apoptotic family members or, to a lesser extent, display loss-of-function mutations of Bax (Garcia *et al.*, 2002; Likui *et al.*, 2009; Meijerink *et al.*, 1998; Pepper *et al.*, 2008) and *in vitro* studies demonstrate that simultaneous treatments are unable to overcome TRAIL resistance-induced by a deficiency of Bax or the overexpression of Bcl-2 (Fulda *et al.*, 2002; LeBlanc *et al.*, 2002; von Haefen *et al.*, 2004). Because recombinant TRAIL or moAb targeting TRAIL-R1 or TRAIL-R2 have been administered simultaneously starting from day 1 of each cycle with the chemotherapeutic compounds of interest, in most if not all clinical studies, the lack of efficacy of these combinations may be attributed to their inability to overcome the mitochondrial block (El Fajoui *et al.*, 2011; Ganten *et al.*, 2004; Jacquemin *et al.*, 2012; Morizot *et al.*, 2011; Ndozangue-Tourigouine *et al.*, 2008; von Haefen *et al.*, 2004).

However, some chemotherapeutic drugs applied sequentially are able to overcome resistance induced by one or even two TRAIL signalling checkpoints, including those acting at the mitochondrial level (Galligan *et al.*, 2005; Ivanov *et al.*, 2007; Morizot *et al.*, 2011; Shankar *et al.*, 2005; Singh *et al.*, 2003). As illustrated in Figure 3, while simultaneous treatment with TRAIL and etoposide (VP16) fails to cooperate to induce apoptosis in the colon cancer cell line HCT116, deficient for Bax (Bax^{-/-}), sequential administration of TRAIL and VP16 overcomes Bax-deficiency (Figure 3, adapted from Morizot *et al.*; 2011). Yet, when Bax deficiency is associated with the ectopic expression of TRAIL-R4, this combination fails to restore apoptosis induced by TRAIL (Figure 3). However, when other chemotherapeutic regimens, such as the metabolic inhibitor 5-FU, are used sequentially, they can afford TRAIL induced cell death restoration in Bax-deficient HCT116 cells expressing TRAIL-R4 ectopically (Figure 3), owing to 5-FU's ability to inhibit c-FLIP expression (Galligan *et al.*, 2005; Morizot *et al.*, 2011). Similarly, in the cervical adenocarcinoma cell line HeLa, sequential treatment with 5-FU and TRAIL can overcome resistance induced by ectopic expression of TRAIL-R4 alone or TRAIL-R4 and Bcl-2, but fails to restore sensitivity to TRAIL-induced cell death when TRAIL-R4 is expressed together with the caspase-8 inhibitor c-FLIP (Figure 3).

It is unclear why sequential treatments are superior to combined treatments and why some chemotherapeutic drugs are able to bypass two checkpoints while others only manage to circumvent one at a time. Nonetheless, similar concepts have recently been documented for targeted therapies combined with DNA-damaging agents. It has been demonstrated for example that time-staggered EGFR inhibition, but not simultaneous coadministration, sensitized triple-negative breast cancer cells to genotoxic drugs (Lee *et al.*, 2012). As far as TRAIL is concerned, we and others have demonstrated that sequential treatments with some therapeutic agents induce an increase in DISC formation and caspase-8 activation at the membrane (Ganten *et al.*, 2004; Lacour *et al.*, 2003; Morizot *et al.*, 2011), while others, including polyphenol derivatives or oxaliplatin act mainly at the mitochondrial level (El Fajoui *et al.*, 2011; Jacquemin *et al.*, 2012). Some compounds, including the metabolic inhibitor 5-FU are able to enhance DISC formation and inhibit c-FLIP at the same time (Morizot *et al.*, 2011). As a matter of fact, inhibition of c-FLIP expression by 5-FU requires much more time than TRAIL to induce DISC formation and caspase activation, which takes place within minutes. Thus, simultaneous stimulations are unlikely to provide enough time to inhibit c-FLIP expression or to allow molecular events leading to enhanced TRAIL DISC formation. This increase in DISC formation and in

caspase-8 activation by some chemotherapeutic compounds is essential to bypass the mitochondrial block (Morizot *et al.*, 2011; Ndozangue-Touriguine *et al.*, 2008). Alternatively, many chemotherapeutic compounds have been described to enhance TRAIL-induced cell death mediated by up-regulating TRAIL-R1 or TRAIL-R2 (Baritaki *et al.*, 2007; David *et al.*, 2008; Ding *et al.*, 2011; Hori *et al.*, 2010; Kwon *et al.*, 2008; Liu *et al.*, 2007; Son *et al.*, 2007; Zhu *et al.*, 2010).

The hope that TRAIL or its derivatives could be used as single agents to treat patients suffering from cancer is clearly over, but there is still a possibility that TRAIL-based antitumoral therapies, in association with targeted or conventional chemotherapy, will be of interest in oncology. Understanding more precisely the molecular mechanisms underlying TRAIL signalling checkpoint regulation by conventional chemotherapy or targeted anti-cancer compounds, is required more than ever before designing novel clinical trials aiming at evaluating the efficacy of these PARAs in the clinic. Whatever the molecular mechanism required, future clinical studies, besides monitoring TRAIL receptor, c-FLIP and Bcl-2 family members expression levels, in order to select patients that may mostly benefit from these combinations, should also consider administrating TRAIL sequentially after chemotherapy, to overcome resistance induced at the mitochondrial level. Sequential therapies should prove valuable, provided that the combination acts cooperatively and is not deleterious for TRAIL signalling.

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Table 1: Clinical trials of TRAIL and TRAIL derivatives

rhTRAIL					
Phase	Pts n(=)	Patients	Company / Safety	Best Response	Reference
Dulanermin (rhApo2L) or AMG951			Genentech/AMGEN		
la	71	Advanced or metastatic solid tumours	Safe and well tolerated up to 30 mg/kg IV - 1/2 life 1 hour Peak plasmatic concentrations compatible with preclinical studies	SD(33) PR (2/5) chondrosarcoma	Herbst et al. 2010a Ling et al. 2006 Herbst et al. 2006
la	67	Advanced solid tumours or NHL	Population pharmacokinetic : No influence of gender, race, albumin, alkaline phosphatase or aspartate aminotransferase	NR	Xin et al. 2008
la	71	Advanced tumours	Serum caspase 3/7 and gDNA levels were observed in >50% of the pts treated with TRAIL	NA	Pan et al. 2011 Pan et al. 2007
CPT (Circularly Permuted TRAIL)			Sunbio Biotech		
lb	27	Relapsed/refractory multiple myeloma	Safe and well tolerated up to 15 mg/kg IV - 1/2 life 1 hour - Peak plasmatic concentrations compatible with preclinical studies	CR(1), PR(4)	Chen et al. 2012b
II	27		Severe AE in 3 pts - one with CPT-related liver injury	nCR(1), PR(8) ORR 33%	Chen et al. 2012c
MoAb Anti-TRAIL-R1					
Phase	Pts n(=)	Patients	Company / Safety	Best Response	Reference
Mapatumumab or TRM1 or HGS-ETR1			GSK/HGS/Takeda		
la	49	Advanced solid tumours	Safe and well tolerated up to 20 mg/kg IV - 1/2 life 18-21 days Peak plasmatic concentrations compatible with preclinical studies	SD (19)	Tolcher et al. 2007
la	41	Advanced solid tumours		SD (12)	Hotte et al. 2008
lb/ II	40	Relapsed/refractory NHL	3 clinical responses out of 15 follicular lymphoma pts	CR(2) PR(1) SD(12)	NCT00094848 Younes et al. 2010
II	32	Relapsed/refractory stage IIIb/IV or recurrent NSCLC	No AE, but no clinical activity demonstrated.	SD (9)	NCT00092924 Greco et al. 2008
II	38	Refractory colorectal cancer		SD (12)	Trarbach et al. 2010

Table 1: Clinical trials of TRAIL and TRAIL derivatives

MoAb Anti- TRAIL-R2					
Phase	Pts n(=)	Patients	Company / Safety	Best Response	Reference
Conatumumab or AMG655					
AMGEN					
la	37	Advanced solid tumours	Safe and well tolerated up to 20 mg/kg IV - 1/2 life 13-19 days	evidence of activity PR (1 -over 4 years) SD(14)	Herbst et al. 2010b LoRusso et al. 2007
I	18	Advanced solid tumours		SD (9)	Doi et al. 2011
Drozitumab or Apomab or PRO95780					
Genentech					
la	50	Advanced treatment or refractory solid tumours	Tolerated up to 20 mg/kg IV but possible adverse hepatic events in 4 pts	SD (20) 23% tumour mass reduction in 3 pts	Camidge. et al. 2010
II	128*	Untreated, advanced-stage NSCLC	completed	NR	NCT00480831
II	90*	Advanced chondrosarcomas	Efficacy not evidenced for this population	completed	NCT00543712
LBY-135					
Novartis					
I	32	Advanced solid tumours	Safe and well tolerated up to 20 mg/kg IV - 1/2 life 10 days signs of clinical activity	2 pts decreased tumour markers (50% and 40%).	Sharma et al. 2008
Lexatumumab or HGS-ETR2					
HGS/Kirin Brewery Co					
la	37	Advanced solid tumours	Safe and well tolerated up to 10 mg/kg IV - 1/2 life 2-18 days	SD (12)	Plummer et al. 2007
la	31	Advanced solid tumours and lymphoma	Safe and well tolerated up to 10 mg/kg IV	SD(10*) *1 mixed response	Wakelee et al. 2010 Patnaik et al. 2006
I	24	Pediatric solid tumours		SD(5) - Clinical response observed 2 pts	NCT00428272 Merchant et al. 2012
Tigatuzumab or CS-1008 or TRA-8					
Daiichi Sankyo					
I	17	Relapsed/refractory solid tumours or lymphomas	Safe and well tolerated up to 8 mg/kg IV - 1/2 life 8-16 days	SD (7)	NCT00320827 Forero-Torres et al. 2010

* Estimation/expected ; **AE** Adverse Events; **CR** Complete Response; **NA** Not Applicable; **nCR** near Complete Response; **NHL** Non Hodgkin Lymphoma; **NR** Not Reported; **ORR** Objective Response Rate; **PR** Partial Response; **pts** patients; **SD** Stable Disease.

Table 2: Clinical trials associating TRAIL and chemotherapy

Dulanermin or AMG951						
Combination	Phase	Pts n(=)	Tumour	Safety	Best Response	Reference
Rituximab	Ib	7	low-grade NHL	combination appears safe and shows evidence of activity	CR(2) PR(1) SD(1)	NCT00400764 Yee <i>et al.</i> 2007 Belada <i>et al.</i> 2010
	II R	48		NOT better than Rituximab alone		
Caboplatin, Paclitaxel & Bevacizumab	Ib	24	Advanced tumours	Dulanermin plus PCB was well tolerated with no occurrence of DLT	CR(1) PR(13) SD(9)	Soria <i>et al.</i> 2010 NCT00508625 Blackhall <i>et al.</i> 2010 Soria <i>et al.</i> 2011
	Ib & II	213	Untreated advanced stage IIIb/IV NSCLC	NOT better than PC or PCB PK appeared unaltered		
FOLFOX Bevacuzimab	Ib	23	Untreated, locally advanced, recurrent, or mCRC	No adverse interactions	PR(12 ± 3*) SD(7)	NCT00873756 Kozloff <i>et al.</i> 2012
AMG479 (anti-IGFR1)	1b/II	89*	Advanced refractory solid tumours (NSCLC, CRC pancreatic ovarian & sarcomas)	NR	Terminated	NCT00819169
Irinotecan & Cetuximab OR FOLFIRI	Ib	30	mCRC	Safe with Irinotecan-regimen	NR	Yee <i>et al.</i> 2009
Camptosar & Erbitux or FOLFIRI w/o Bevacizumab	Ib	NR	Previously treated mCRC	NR	Ongoing, not recruiting	NCT00671372
FOLFIRI Bevacuzimab	Ib	27		Safe with FOLFIRI (± Bevacuzimab)	PR(6) SD(17)	Kasubhai <i>et al.</i> 2012

CPT						
Combination	Phase	Pts n(=)	Tumour	Safety	Best Response	Reference
Thalidomide	II	43	Relapsed/refractory multiple myeloma	Safe with thalidomide Better than thalidomide alone	CR(2), nCR(3), PR(4)	Chen <i>et al.</i> 2012a

* Estimation/expected ; **Camptosar** irinotecan HCL injection; **CR** Complete Response; **CRC** Colorectal Cancer; **FOLFIRI** Folinic acid, Fluorouracil, Irinotecan; **FOLFOX** Folinic acid, Fluorouracil, oxaliplatin; **NHL** Non Hodgkin Lymphoma; **NR** Not Reported; **NSCLC** Non Small Cell Lung Carcinoma; **PCB** Paclitaxel, Carboplatin, Bevacizumab; **PK** Pharmacokinetics; **PR** Partial Response; **R** Randomized; **SD** Stable Disease.

Table 3: Clinical trials associating Mapatumumab and chemotherapy

Mapatumumab						
Combination	Phase	Pts n(=)	Tumour	Safety	Best Response	Reference
Paclitaxel & Caboplatin	I	27	Advanced Solid tumours	Safe with Paclitaxel & Cisplatin up to 20 mg/kg with no occurrence of DLT	PR(5) SD(12)	Leong <i>et al.</i> 2009
	Ib	28	Advanced solid tumours	PK profile of HGS-ETR1 not affected by paclitaxel and carboplatin	PR(6) SD(13)	Chow <i>et al.</i> 2006
Paclitaxel & Caboplatin	II R	111	First line Advanced NSCLC	The results do not support further evaluation in combination with PC in pts with advanced NSCLC	similar to PC alone	NCT00583830 Von Pawel <i>et al.</i> 2011
Gemcitabine and Cisplatin	Ib	49	Advanced solid tumours	Safe with Gemcitabine & Cisplatin at doses up to 30 mg/kg	PR(12) SD(25)	Mom <i>et al.</i> 2009
Cisplatin & Radiotherapy	Ib/II	42*	First Line Advanced CervicalCancer	recruiting	NR	NCT01088347
Sorafenib	Ib	19	Advanced hepatocellular carcinoma & chronic viral hepatitis	Safe with Sorafenib at doses up to 30 mg/kg	PR(2) SD(4)	NCT00712855 Sun <i>et al.</i> 2011
	II	100*	First line Advanced Hepatocellular Carcinoma	Ongoing	NR	NCT01258608
Bortezomib	II R	104	Relapsed/refractory multiple myeloma	No Adverse effects but no benefit	similar to Bortezomib alone	NCT00315757 Belch <i>et al.</i> 2011

* Estimation/expected ; **CR** Complete Response; **CRC** Colorectal Cancer; **DLT** Dose Limiting Toxicity; **FOLFIRI** Folinic acid, Fluorouracil, Irinotecan; **FOLFOX** Folinic acid, Fluorouracil, oxaliplatin; **mCRC** metastatic Colorectal carcinomas; **NHL** Non Hodgkin Lymphoma; **NR** Not Reported; **NSCLC** Non Small Cell Lung Carcinoma; **ORR** Objective Response Rate; **PCB** Paclitaxel, Carboplatin, Bevacizumab; **PR** Partial Response; **R** Randomized; **SD** Stable Disease.

Table 4: Clinical trials associating anti-TRAIL-R2 antibodies and chemotherapy

Conatumumab						
Combination	Phase	Pts n(=)	Tumour	Safety	Best Response	Reference
Ganitumab (or AMG 479)	I	9	Advanced, refractory solid tumours	Safe with AMG 479 up to 15 mg/kg	SD(3)	NCT00819169 Chawla <i>et al.</i> 2010
Doxorubicin	II R	128	Unresectable soft tissue sarcomas	Safe with doxorubicin up to 15 mg/kg Not better than doxorubicin alone	ORR decreased vs placebo 20 vs 24%	NCT00626704 Demetri <i>et al.</i> 2012
mFOLFOX6 & Bevacizumab	Ib/2	202*	First-Line mCRC	NR	Ongoing, not recruiting	NCT00625651
FOLFIRI or ganitumab + FOLFIRI	II R	155	Second-line treatment mutant KRAS mCRC	CON+F tolerable in this population CON + F improved PFS	ORR was increased vs Placebo 14 vs 2%	NCT00813605 Cohn <i>et al.</i> 2012
Gemcitabine or Gemcitabine + AMG 479	II R	125	Metastatic pancreatic cancers	Safe with Gemcitabine up to 10 mg/kg	Some activity PR(1) SD(22)	NCT00630552 Kindler <i>et al.</i> 2012
Caboplatin & Paclitaxel	Ib/2	172*	First-Line advanced NSCLC	NR	Completed	NCT00534027
Panitumumab	Ib/2	53*	mCRC	NR	NR	NCT00630786
Bortezomib or Vorinostat	Ib	62*	Relapsed or refractory lymphomas	NR	Suspended	NCT00791011

Drozitumab						
Combination	Phase	Pts n(=)	Tumour	Safety	Best Response	Reference
Rituximab	II	40	Relapsed NHL	Safe with Rituximab up to 15 mg/kg. Not better than Rituximab alone	CR(2) PR(18)	NCT00517049 Wittebol <i>et al.</i> 2010
Caboplatin, Paclitaxel ± Bevacuzimab	II R	124	Previously untreated stage IIIb, IV NSCLC	The results do not support further evaluation with PC ± B	similar to PC± B alone	Karapetis <i>et al.</i> 2010
Cetuximab & Irinotecan OR FOLFIRI ± Bevacuzimab	Ib	20	First line mCRC	Safe with Irinotecan-regimens up to 15 mg/kg	PR(3*) SD(13)	NCT00497497 Baron <i>et al.</i> 2011
FOLFOX ± Bevacuzimab	Ib	9	First line mCRC	The results do not support further evaluation with FOLFOX ± B	PR(5*) SD(3)	NCT00851136 Rocha Lima <i>et al.</i> 2011 & 2012

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Combination	Phase	Pts n(=)	Tumour	Safety	Best Response	Reference
Capecitabine	I	24	Advanced solid tumours	Tolerated with capecitabine at 20 mg/kg but 2 grade 3 DLTs observed at 1 and 20 mg/kg	Some activity PR(1) - 2 pts with decreased tumour vol. 60-73%	Sharma <i>et al.</i> 2008

Lexatumumab

Combination	Phase	Pts n(=)	Tumour	Safety	Best Response	Reference
Gemcitabine, pemetrexed, Doxorubicin or FOLFIRI	Ib	41	Advanced solid tumours	Tumour shrinkage observed, confirmed partial responses (PRs) in the FOLFIRI and doxorubicin arms	NR	Sikic <i>et al.</i> 2007
IFN γ	I	19	Refractory pediatric solid tumours		NR	NCT01445093

Tigatuzumab

Combination	Phase	Pts n(=)	Tumour	Safety	Best Response	Reference
Caboplatin, Paclitaxel	II R	109*	Metastatic or unresectable NSCLC	NR	Completed	NCT00991796
	II	24	Locally advanced or metastatic ovarian cancer	NR	Completed	NCT00945191
Gemcitabine	II	65*	Untreated and unresectable pancreatic cancer	NR	Completed	NCT00521404
Irinotecan	II R	8*	mCRC failed first-line treatment with oxaliplatin	NR	Completed	NCT00969033
FOLFIRI	I	20*	Patients who have failed other treatments	NR	Ongoing, not recruiting	NCT01124630
Sorafenib	II R	160*	Advanced liver cancer	NR	Ongoing, not recruiting	NCT01033240
Abraxane (Paclitaxel)	II R	60*	Metastatic triple negative breast cancer	NR	Ongoing, not recruiting	NCT01307891

Estimation/expected ; **CR** Complete Response; **CRC** Colorectal Cancer; **DLT** Dose Limiting Toxicity; **FOLFIRI** Folinic acid, Fluorouracil, Irinotecan; **FOLFOX** Folinic acid, Fluorouracil, oxaliplatin; **mCRC** metastatic Colorectal carcinomas; **NHL** Non Hodgekin Lymphoma; **NR** Not Reported; **NSCLC** Non Small Cell Lung Carcinoma; **ORR** Objective Response Rate; **PCB** Paclitaxel, Carboplatin, Bevacizumab; **PR** Partial Response; **R** Randomized; **SD** Stable Disease.

Figure legends:

Figure 1 : Simplified schematic representation of Fas ligand, TNF- α and TRAIL-induced signalling : (A) Binding of TNF- α to TNF-RI induces the formation of a membrane bound-complex, composed of RIPK1, TRAF-2, TRADD, IAPs, LUBAc and IKKs, that mainly triggers NF- κ B activation and cell survival through the transcriptional regulation of the caspase-8 inhibitor c-FLIP. From complex I, a pro-apoptotic cytosolic complex (complex II) is generated, containing the initiator caspase-8 and the adaptor protein FADD. Complex II formation is partly regulated by the linear ubiquitin chain assembly complex, LUBAc, which contains HOIP, HOIL-1 and Sharpin and its pro-apoptotic function is inhibited by c-FLIP. Since the vast majority of cells are proficient for NF- κ B activation, upon TNF- α stimulation, TNF-RI fails most of the time to trigger apoptosis. (B) TRAIL- and Fas ligand are potent apoptotic inducers. Binding of these ligands to their cognate pro-apoptotic receptors, TRAIL-R1 or TRAIL-R2 and Fas, respectively, trigger the formation of a membrane complex coined DISC (Death-Inducing Signalling Complex), in which the adaptor protein FADD and the pro-caspase-8/-10 are recruited allowing strong caspase activation and apoptosis triggering. Apoptosis induced by these ligands is either induced through direct caspase-8-mediated caspase-3 activation, or through an amplification loop involving the mitochondria and the cleavage of the BH3-only protein Bid by caspase-8.

Figure 2 : Fas ligand and TRAIL recombinant proteins or derivatives assessed in clinical trials: Schematic representation of (A) Fas ligand, TRAIL recombinant preparations and TRAIL agonistic monoclonal antibodies assessed in clinical trials and (B) the 4 main receptors to which TRAIL can bind, namely TRAIL-R1, TRAIL-R2, TRAIL-R3 and TRAIL-R4. (C) Table representing potential proapoptotic capabilities of TRAIL preparations or monoclonal antibodies targeting TRAIL-R1 or TRAIL-R2 in tumour cells expressing variable amounts of TRAIL receptors. Receptors represented in light colour indicate poor engagement of the apoptotic machinery. S stands for potentially sensitive tumour cells; R, potentially resistant tumour cells; S/R, cells potentially sensitive or resistant to TRAIL-induced apoptosis depending on the selective or preferential engagement by TRAIL-R1 or TRAIL-R2. (D) Schematic representation of receptor complex formation and apoptosis induced by TRAIL recombinant preparations or monoclonal antibodies targeting TRAIL-R1 or TRAIL-R2. In all cases, overexpression of Bcl-2 family anti-apoptotic members or c-FLIP by tumour cells may impair TRAIL-induced cell death. TRAIL-R3 or TRAIL-R4 expression by tumour cells on the other hand can only impair apoptosis-induced by recombinant TRAIL preparations.

Figure 3 : Differential TRAIL-induced apoptosis following combined vs sequential chemotherapy. (A) Schematic representation of the treatment protocols used panel B. (B) TRAIL-induced apoptosis in HCT116 WT cells (Empty squares), HCT116 Bax deficient (Bax^{-/-}) (Grey squares) or HCT116 Bax deficient expressing ectopically TRAIL-R4 (Bax^{-/-}(TRAIL-R4)) cells (Black squares), stimulated either sequentially with etoposide (VP16) or simultaneously (Combo). For sequential treatments, cells were first incubated for 3 hours in the presence of 10 μ M VP16, washed, allowed to recover at 37°C for 45 hours and then stimulated with 500 ng/ml TRAIL for 6 hours. Alternatively, cells were stimulated simultaneously with TRAIL and VP16 (combo), or with single agents for 24 or 48 hours respectively. Apoptosis was measured by Hoechst staining. (C) Schematic representation of the treatment protocols used panel D. (D) Apoptosis induced by TRAIL, 5-FU or sequential treatments associating 5-FU and TRAIL in HeLa WT cells (Empty squares) or HeLa cells expressing TRAIL-R4 ectopically (Empty red squares), TRAIL-R4 and Bcl-2 (Grey squares) or TRAIL-R4 and c-FLIP (Black squares). HeLa cells were stimulated or not for 72 hours with 1 μ M 5-FU, then treated or not with 500 ng/ml TRAIL for 6 hours and apoptosis was monitored by Hoechst staining. Modified from Morizot et al. (2010).