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Smac mimetics and TNFα: a dangerous liaison?

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Summary

Inhibitor of apoptosis proteins (IAPs) such as XIAP, cIAP1 and cIAP2 are upregulated in many cancer cells. It has been thought that small molecule mimetics of Smac, an endogenous IAP antagonist, might potentiate apoptosis in cancer cells by promoting caspase activation. However, three recent papers in Cell (Vince et al., 2007; Varfolomeev et al., 2007) and Cancer Cell (Petersen et al., 2007) now report that Smac mimetics primarily kill cancer cells via a different mechanism, the induction of autoubiquitination and degradation of cIAPs, which culminates in TNF α -mediated cell death. These studies may accelerate the introduction of Smac mimetics in cancer therapy.

Re-establishing the apoptotic program is a much dreamed about strategy for cancer therapy. One possible approach involves small molecules that mimic the antagonistic activity of Smac (also known as Diablo) towards inhibitor of apoptosis (IAP) proteins. Smac and its synthetic small molecule mimetics interact with the BIR domains in IAPs such as XIAP, cIAP1, and cIAP2. Because XIAP is a bona fide caspase inhibitor, it was presumed that Smac mimetics primarily target XIAP and therefore allow caspase-9 activation to occur to trigger apoptosis. In contrast to XIAP, cIAP1 and cIAP2 are weak caspase inhibitors *in vitro*, but associate with two tumor necrosis factor receptor (TNFR) signaling proteins TRAF1 and TRAF2 to participate in TNF α -induced NF- κ B activation (Rothe et al., 1995). It has been hoped that Smac mimetics could be used in combination with chemo- or radiation therapy that activates caspase-9 via the mitochondrial apoptotic pathway. In principal, this combination therapy should enhance cancer cell apoptosis by relieving any caspase activities held in check by IAPs.

In three related papers by Vucic, Wang, Silke and their colleagues, Smac mimetics exceed expectations as potentiators of cancer cell apoptosis by exhibiting the ability to induce cell death in multiple cancer cell lines even when used alone (Petersen et al., 2007; Varfolomeev et al., 2007; Vince et al., 2007). The three groups independently developed small molecules that mimic the N-terminal AVPI sequence of mature Smac but are different in their detailed chemical structures. All but one of the developed Smac mimetics are dimeric, resembling the dimeric structure of Smac. As evidence that this killing activity is likely not due to off-target toxicity, the Smac mimetic used by Petersen et al. (2007) only pulled down its intended targets XIAP, cIAP1, and cIAP2, and their associated proteins TRAF1 and TRAF2. Surprisingly, cell death induced by the Smac mimetics is dependent on caspase-8, but not caspase-9 (Petersen et al., 2007; Varfolomeev et al., 2007) and this cell death can be prevented by the caspase-8 inhibitor crmA (Vince et al., 2007). Because caspase-8 is the primary initiator caspase in the apoptotic pathway mediated by death receptors (such as Fas, TRAIL receptors, and TNF receptor 1) the dependence on caspase-8 is consistent with the

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Elegant independent studies by the three groups have now uncovered TNF α , a death receptor ligand, as the major culprit in cancer cell apoptosis induced by Smac mimetics (Petersen et al., 2007; Varfolomeev et al., 2007; Vince et al., 2007). siRNA knockdown of TNF receptor 1 (TNFR1) but not of Fas, TRAIL-R1 and TRAIL-R2 prevented Smac mimetic-induced apoptosis of cancer cell. Similarly, specific knockdown of TNF α but not FasL or TRAIL promoted the survival of cells treated with Smac mimetics (Petersen et al., 2007; Varfolomeev et al., 2007). Thus, the emerging model (Figure 1) is that in addition to targeting XIAP to relieve caspase-9 inhibition in the intrinsic cell death pathway, Smac mimetics target cIAPs, especially cIAP1, to stimulate their auto-ubiquitination and degradation, leading to NF- κ B activation and TNF α secretion. The autocrine TNF α signaling in turn induces caspase-8 activation and cancer cell death.

The first dramatic effect of Smac mimetics is the induction of cIAP1 and cIAP2 degradation within minutes of treatment without affecting the levels of other IAPs or TRAFs (Varfolomeev et al., 2007; Vince et al., 2007). The degradation of cIAP1 and cIAP2 is dependent on the concentration of the Smac mimetic, with cIAP1 degradation occurring at a much lower concentration than cIAP2. Because cIAPs are ubiquitin ligases capable of auto-ubiquitination and ubiquitination of several binding proteins, it was suspected that their degradation might depend on the proteasome. Indeed, the proteasome inhibitor MG132 prevented the loss of cIAP1 and cIAP2 induced by the Smac mimetic (Varfolomeev et al., 2007; Vince et al., 2007). In contrast, the pan-caspase inhibitor z-VAD did not have any effect on cIAP1 and cIAP2 degradation. For Smac mimetic-induced degradation to occur, the IAP proteins require both a functional RING domain and an intact interaction site for a Smac mimetic. Furthermore, *in vitro* reconstitution of the auto-ubiquitination reaction using purified E1, E2 (UbcH5a) and cIAP1 showed that Smac mimetics quickly and markedly enhanced auto-ubiquitination of cIAP1 (Varfolomeev et al., 2007).

How Smac mimetics stimulate cIAP1 auto-ubiquitination remains unclear. Although a bivalent Smac mimetic induces dimerization of the cIAP1 BIR2-BIR3 domain *in vitro*, this dimerization is not a prerequisite for cIAP1 ubiquitination and degradation, as the monovalent Smac mimetic is equally effective at similar concentrations (Varfolomeev et al., 2007). Another possibility is offered by the fact that many ubiquitin ligases stimulate their own ubiquitination in the absence of their substrates. However, the Smac mimetics did not dissociate cIAPs from TRAF2, and hence cIAPs likely stay bound with TRAF2-associated ubiquitination targets (Varfolomeev et al., 2007). At the moment, the most plausible explanation is that Smac mimetics alter the protein conformation of cIAPs to allow better access of the Lys residues to the E2s bound to the RING domain.

Loss of cIAPs induces activation of both the canonical and non-canonical NF- κ B signaling pathways as manifested by I κ B degradation and p100 processing, respectively. Remarkably, I κ B phosphorylation occurs within minutes of treatment of Smac mimetics, with kinetics that are similar to TNF α -induced NF- κ B activation. I κ B phosphorylation and degradation is not affected by co-treatment with an inhibitor of TNFR1 signaling, demonstrating that receptor signaling is not involved in activation of the canonical NF- κ B pathway by the Smac mimetic (Varfolomeev et al., 2007). An increase in the recruitment of the kinase RIP1 (receptorinteracting protein 1) to the TNFR1 signaling complex was observed after short treatment of a Smac mimetic (Vince et al., 2007). Increased RIP1 recruitment was also observed in cIAP1-deficient mouse embryonic fibroblasts (MEFs), which cannot be further increased by Smac mimetic treatment (Vince et al., 2007), suggesting that loss of cIAP1 is responsible for the increased recruitment of the RIP1. Because RIP1 binding to TNFR1 is known to result in NF- κ B activation, it is argued that loss of cIAP1 leads to activation of the canonical NF- κ B pathway via increased RIP1 recruitment to TNFR1 (Vince et al., 2007). However, because the pull down establishing enhanced RIP1 recruitment was performed with Fc-TNF α and not with an antagonistic antibody to TNFR1, the possibility exists that the increased RIP1 recruitment is dependent on TNF α , rather than a direct effect of the Smac mimetics.

The mechanism for the activation of the non-canonical NF-κB pathway by Smac mimetics is more clearly elucidated (Varfolomeev et al., 2007; Vince et al., 2007). A highly labile kinase NIK is a crucial player in this pathway. It phosphorylates IKK α , leading to p100 phosphorylation and processing to p52. NIK constitutively associates with TRAFs, such as TRAF2 and TRAF3, which have been shown to negatively regulate the non-canonical NFκB pathway (He et al., 2007; Malinin et al., 1997). Because TRAF2 in turn forms a constitutive complex with cIAPs, it was hypothesized that cIAPs may be the ubiquitin ligase for NIK ubiquitination and responsible for the instability of NIK in cells. Indeed, coexpression of NIK with cIAP1 or cIAP2 led to disappearance of NIK whereas co-expression with a RING domain mutant of cIAP1 did not. Treatment of cells stably expressing NIK with a Smac mimetic triggered degradation of endogenous cIAP1 and a remarkable increase of the NIK protein level. The Smac mimetic-induced processing of p100 occurs with much slower kinetics than IkB phosphorylation, presumably due to the time required to synthesize and to accumulate NIK to a functional level. Degradation of cIAP1 and stabilization of NIK are also components of the physiological non-canonical NF- κ B pathway as shown by treatment with the TNF-like ligand TWEAK (Varfolomeev et al., 2007).

Activation of the NF- κ B signaling pathway leads to the induction of many genes. An important effector protein in this case is TNF α , which is only induced in cancer cell lines sensitive to treatment with Smac mimetics (Varfolomeev et al., 2007; Vince et al., 2007). What determines whether NF- κ B activation induces TNF α production is not known. Sensitive cell lines already secrete TNF α before any treatment (Petersen et al., 2007), although it is unknown whether TNF α secretion is further increased in these same cell lines upon treatment with Smac mimetics. It is also unclear whether only one or both of the canonical and non-canonical NF- κ B pathways is required for TNF α induction. A more indepth analysis will be required to clarify this point.

Remarkably, loss of cIAPs due to Smac mimetic treatment also appears to assist TNF α induced cell death. Unlike FasL, TNF α does not readily induce cell death in most circumstances. Rather, it activates NF- κ B and MAP kinases, leading to cell survival and cell activation. Previous studies have shown that TNF α -induced cell death is accomplished via a secondary TRADD-RIP1-FADD-caspase-8 complex devoid of TNFR1 (Micheau and Tschopp, 2003). In keeping with this notion, RIP1 is also required for cell death induced by a Smac mimetic, even in the presence of exogenous TNF α for both sensitive cancer cell lines and those that are resistant to Smac mimetics alone but do respond to co-stimulation by TNF α (Petersen et al., 2007). In contrast, knockdown of the adaptor protein TRADD somewhat increases apoptosis (Petersen et al., 2007). Immunoprecipitation of caspase-8 after Smac mimetic treatment revealed the formation of a RIP1-FADD-caspase-8 complex that did not contain TRADD or TRAF2 (Petersen et al., 2007). The lack of TRADD and TRAF2 in the death signaling complex is interesting as it is different from the classical view in which TRADD serves as the central platform molecule for the recruitment of both TRAF2 and FADD.

Is it possible that cancer cells respond to TNF α differently? In fact, a recent report also showed that RIP1 is crucial in TNF α -induced cell apoptosis in human tumor cells (Jin and

El-Deiry, 2006). RIP1 was first cloned as a Fas interacting protein that induces apoptosis upon overexpression (Stanger et al., 1995) and was also shown to be essential for FasL-mediated necrotic cell death (Holler et al., 2000). However, RIP1 knockout cells are defective in TNF α -induced NF- κ B activation (Kelliher et al., 1998). The apparent involvement of RIP1 in both apoptosis and activation of NF- κ B induced is consistent with RIP1 as both a protein required for survival and a protein that can induce cell death when overexpressed.

The studies collectively reveal that there may be three different kinds of cancer cells defined by their resistance and sensitivity to Smac mimetics. The first type would be those that are sensitive to Smac mimetics as single agents. In this type of cell, Smac mimetics induce or perhaps enhance TNFa synthesis and secretion and render the cells sensitive to TNFainduced apoptosis by forming a RIP1-dependent caspase-8 activating complex. The second type of cancer cells would be those that do not respond to Smac mimetic treatment alone, but are sensitive to Smac mimetics when co-stimulated by exogenous TNF α . In these cells, treatment with a Smac mimetic fails to induce $TNF\alpha$ secretion, but sensitizes the cells to apoptosis induced by several death receptors. The precise mechanism for this pro-death activity is not known. In fact, the increase in NF-KB activity seen in cells treated with Smac mimetics would suggest that the contrary is more prevalent. However, it is likely that apart from NF-kB, other signaling pathways such as JNK are activated to lead to the degradation of the anti-apoptotic protein cFLIP (Chang et al., 2006). Third, there may be a type of cancer cells that are resistant to co-treatment with Smac mimetics and TNFa. These cells do not form a RIP1-dependent caspase-8 activating complex upon the co-treatment. It is interesting that Smac mimetics induced degradation of cIAPs in all cell lines tested (Varfolomeev et al., 2007), suggesting that additional switch points control the sensitivity to Smac mimetics.

In summary, the role of Smac mimetics in cancer cells appears to be two-fold (Figure 1). First, they stimulate auto-ubiquitination of cIAPs, resulting in their proteasomal degradation. This in turn leads to NIK stabilization and facilitates RIP1 recruitment. This results in the activation of the non-canonical and canonical NF- κ B pathways, causing TNF α production in a substantial number of tumor cells. Second, Smac mimetics lead to sensitivity to TNF α -induced cell death, likely through the degradation of cIAPs and by favoring the formation of a RIP1-dependent caspase-8 activating complex. Further boosting the potential of Smac mimetics for clinical use, Petersen et al., (2007) showed that cancer cells that are sensitive to Smac mimetic treatment in vitro are also responsive to the same treatment in an in vivo mouse model.

One might argue, however, that cIAPs are also present in non-transformed cells and that Smac mimetics will be potentially toxic considering the rapid degradation of cIAP1 and activation of NF- κ B in primary MEFs upon Smac mimetic treatment (Vince et al., 2007). Yet, Smac mimetics do not kill non-transformed cells even though increased RIP1 recruitment is observed. Moreover, cIAP1 knockout mice appear normal with respect to TNF α -mediated NF- κ B activation and cell death indicating a high selectivity for tumor cells. It has been known for many years that cell transformation shifts the balance in death receptor signaling pathways from pro-survival to pro-cell death. For example, TRAIL and FasL preferentially kill transformed cells and spare most primary cells. Although the molecular reason for this difference in sensitivity is still poorly understood, this absence of knowledge does not impede the clinical trials that are ongoing with both of the TNF ligand family members.

Ironically, TNF α was the first member of this family to be considered as an antitumor drug. However, despite incredible expectations, the use of TNF α in the treatment of cancer patients turned out to be limited to limb perfusion due to TNF α 's toxic proinflammatory

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activity when administered systemically. This may be set to change with the arrival of Smac mimetics that trigger an increase in TNF α production at a barely detectable and non-toxic level. The late William Coley, who first used stimulation of TNF α production by bacterial infection to induce tumor regression *in vivo* in the late 1800s (Coley, 1893), would have been very pleased.

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Figure 1. Cancer cell death induced by Smac mimetics

In non-stimulated cells, cIAPs bound to TRAF2 ubiquitinate and degrade the kinase NIK thereby inhibiting the non-canonical NF- κ B pathway. The addition of Smac mimetics stimulates auto-ubiquitination of cIAPs, resulting in their proteasomal degradation, which in turn leads to the stabilization of NIK. Moreover, TRAF2 "freed" from cIAPs facilitates recruitment of the kinase RIP1 to tumor necrosis factor receptor 1 (TNFR1). This results in the activation of the non-canonical and canonical NF- κ B pathways, causing TNF α production in a substantial number of tumor cells. Smac mimetics also lead to sensitivity to TNF α -induced cell death, likely through the degradation of cIAPs, which are inhibitors of apoptosis, and by favoring the formation of a RIP1-dependent caspase-8 activating complex.