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Author(s)	Szegezdi, Eva; Mahalingam, Devalingam
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## Kinetics in signal transduction pathways involving promiscuous oligomerizing receptors can be determined by receptor specificity: Apoptosis induction by TRAIL

Eva Szegezdi<sup>1,a</sup>, Almer M. van der Sloot<sup>2,a</sup>, Devalingam Mahalingam<sup>1</sup>, Lynda O'Leary<sup>1</sup>, Robbert H. Cool<sup>3</sup>, Inés G. Muñoz<sup>5</sup>, Guillermo Montoya<sup>4</sup>, Wim J. Quax<sup>3</sup>, Steven de Jong<sup>5</sup>, Afshin Samali<sup>1,b</sup> and Luis Serrano<sup>2,6,b</sup>

<sup>1</sup>School of Natural Sciences, National University of Ireland, Galway, Ireland; <sup>2</sup>EMBL/CRG Systems Biology Research Unit, Centre for Genomic Regulation (CRG), UPF, C/ Dr. Aiguader 88, 08003 Barcelona, Spain; <sup>3</sup>Department of Pharmaceutical Biology, University of Groningen, Antonius Deusinglaan 1, 9713 AV, Groningen, the Netherlands; <sup>4</sup>Structural Biology and Biocomputing Programme, Spanish National Cancer Research Centre (CNIO), Macromolecular Crystallography Group, Melchor Fernandez. Almagro 3, 28029 Madrid, Spain; <sup>5</sup>Medical Oncology, University Medical Center Groningen, Hanzeplein 1, 9713 GZ Groningen, the Netherlands; <sup>6</sup>ICREA, Centre for Genomic Regulation (CRG), UPF, C/ Dr. Aiguader 88, 08003 Barcelona, Spain.

<sup>a</sup>: These authors have contributed equally

### <sup>b</sup>All correspondence should be addressed to:

afshin.samali@nuigalway.ie

luis.serrano@crg.es

Running title: Specificity of promiscuous ligands regulates signaling kinetics

#### Abstract:

Here we show by computer modeling that kinetics and outcome of signal transduction in case of heterooligomerizing receptors of a promiscuous ligand largely depend on the relative amounts of its receptors. Promiscuous ligands can trigger the formation of non-productive receptor complexes, which slows down the formation of active receptor complexes and thus can block signal transduction. Our model predicts that increasing the receptor-specificity of the ligand without changing its binding parameters should result in faster receptor activation and enhanced signaling. We experimentally validated this hypothesis using the cytokine Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL) and its four membrane-bound receptors as an example. Bypassing ligand-induced receptor hetero-oligomerization by receptor-selective TRAIL variants enhanced the kinetics of receptor activation and augmented apoptosis. Our results suggest that control of signaling pathways by promiscuous ligands could result in apparent slow biological kinetics and blocking signal transmission. By modulating the relative amount of the different receptors for the ligand, signaling processes like apoptosis can be accelerated or decelerated and even inhibited. It also implies that more effective treatments using proteins therapeutics could be achieved simply by altering specificity.

*Keywords*: apoptosis / death receptor /mathematical modeling / receptor activation kinetics / Tumor necrosis factor-related apoptosis-inducing ligand

#### Introduction

To enable diverse biological responses, many ligand-receptor systems consist of multiple receptors and/or ligands (1-4). Predicting the biological response in such a multi-component system can be complex: the presence of the receptors and ligands does not guarantee that the signal is transmitted. The response can be dictated by the final equilibrium distribution of the ligand with the different receptors, but it is equally possible that the biological effect is more dynamically driven by temporal kinetic effects of the ligand interacting with its receptors (5). This may be in particular the case when the ligand has a short half-life time. In fact, there is evidence that kinetics could play an important role in the outcome of a signal (6). In a simple network, an interaction with similar  $K_d$  but having different  $k_{on}$  and  $k_{off}$  values, could respond differently with a short, or a long pulse of a ligand. In these multi-component systems mathematical modeling is an invaluable tool to understand and predict the outcome of the signaling events (6).

In principle, excluding well-known effects like macromolecular crowding or protein immobilization, one could assume that it should be possible to extrapolate kinetic and equilibrium constants from *in vitro* to *in vivo* conditions, provided that the *in vitro* conditions reflect the *in vivo* ones. However, this simple assumption could fail if the molecule that triggers a signal can be trapped by non-productive interactions that equilibrate slowly with the productive complex. This can significantly slow down or even block the progression of a signal. In such cases one could imagine that increasing the specificity of the ligand towards the productive complex could result in faster kinetics of receptor activation (7). Moreover, if this is the case, it could affect the way many signal transduction pathways are analyzed and simulated since it is conceivable that this scenario could be very common. To explore this possibility, we have selected a relatively well-understood signal transduction pathway that involves both a promiscuous ligand and non-productive receptor ligand complex formation, the Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) induced apoptotic pathway (8). TRAIL is involved in tumor immune surveillance and is an attractive candidate as an anti-cancer therapeutic as it induces apoptosis in a wide range of tumor cells, but not in non-transformed cells (9). TRAIL has five receptors: death receptor-4 (DR4, TRAIL-R1), death receptor-5 (DR5, TRAIL-R2), decov receptor-1 (DcR1, TRAIL-R3), decov receptor 2 (DcR2, TRAIL-R4) and a secreted receptor, osteoprotegerin (OPG). TRAIL induces apoptotic cell death via binding to DR4 or DR5 (10). Upon binding TRAIL, the receptors recruit the adaptor molecule Fas Associated Death Domain (FADD) to their cytoplasmic death domains (DD). Pro-caspase-8, the apical enzyme of the apoptosis-inducing proteolytic enzyme cascade, then binds to FADD, dimerizes and becomes active. Active caspase-8 induces apoptosis by activating downstream members of the protease cascade, such as caspase-3, -6, -7 (type I apoptosis)(11), or by cleaving and activating a pro-apoptotic, Bcl-2 protein family member, Bid, which triggers cytochrome crelease from the mitochondria and thus amplifying the apoptotic trigger by activating the intrinsic pathway of apoptosis (type II apoptosis)(12). It is broadly accepted, that lower levels of caspase-8 triggers apoptosis through the type II pathway (10, 13). In numerous tumors however, the intrinsic pathway of apoptosis is blocked by overexpression of anti-apoptotic Bcl-2 proteins, or the caspase-9 and -3 inhibitor, X-linked apoptosis inhibitor protein (XIAP). Consequently, if the level of procaspase-8 activation fails to reach a certain threshold, the short half-life of active caspase-8 (approximately 20 minutes) in combination with the various anti-apoptotic molecules present in the cell will block the progression of the apoptotic signal and the cell will not commit to death (14, 15). Thus, a timely and efficient pro-caspase-8 activation is a key factor of TRAIL-induced apoptosis (15-17).

Binding of TRAIL to its other three receptors does not induce apoptosis (18). OPG is a secreted, soluble receptor and has a negative regulatory role in TRAIL signaling by sequestering TRAIL from DR4 and DR5. In contrast, DcR1 and DcR2 are membrane bound and transmembrane receptors, respectively. Neither DcR1 nor DcR2 can recruit FADD upon TRAIL binding, therefore they are believed to act as decoy receptors (18) and overexpression of DcR1 or DcR2 has been shown to inhibit TRAIL-induced apoptosis (19-21). The mechanism by which DcR1 and DcR2 block TRAIL-induced apoptosis is thought to involve not only sequestration of TRAIL from DR4 and DR5, but also by formation of inactive, heteromeric complexes with DR4 or DR5 upon exposure to TRAIL (ligand dependent hetero-oligomerization) (20). Decoy receptors have also been suggested to hetero-oligomerize with DR4 and DR5 in a ligand-independent fashion through their pre-ligand assembly domains (PLAD) present on the N terminus of the receptors (22-25). It is unclear, however, what the individual contributions of the various mechanisms in this process are. Given the fact that TRAIL has a much lower affinity for its decoy receptors than for DR5(26), a sequestration-only mechanism-in which decoy receptors simply act as a TRAIL sink-will not work when similar amounts of receptor are present. Likewise, with the ligand-dependent or ligandindependent DcR2/DR4/5 hetero-oligomerization the equilibrium distribution between the receptors will not be changed much either due to these affinity differences and hence cannot be responsible for observed antagonistic effects either.

Using biochemical data from the literature and this study we have modelled the activation of the TRAIL receptors upon binding TRAIL using SmartCell (27). Our simulations suggest that the receptor towards which the ligand has the highest specificity — but without necessarily having increased kinetic binding constants— will be activated much faster than the other receptors and will

therefore be the main determinant of the signal transduction pathway activated. To verify if this prediction holds in cells, we have used a TRAIL variant that selectively binds to DR5 (D269H/E195R) (28). This TRAIL variant has proved to be a very potent inducer of apoptosis in tumor cell lines where the DR5 receptor is active both *in vitro* (28) and *in vivo* (29). Using this TRAIL variant as a tool we found that receptor-specific TRAIL variants exhibit much faster receptor activation kinetics confirming the prediction of the model. We also show that this multi-receptor system works in concert to create a temporal control of apoptosis induction and, under physiological conditions, can act as a safeguard against unwarranted induction of apoptosis. Thus our results indicate that kinetics of signal transduction induced by promiscuous ligands are largely determined by non-productive interactions and consequently, by the relative amount of the different receptors sharing the same ligand.

#### **Materials and Methods**

#### **Cell Culture and treatments**

Colo205 cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FBS), 2 mM L-glutamine, 50 U/ml penicillin, 5 mg/ml streptomycin and 10 mM sodium pyruvate. Cells were treated with recombinant human TRAIL (TRAIL; non-tagged, amino acids 114-281 fragment), DR5-specific TRAIL variant, D269H/E195R. WT TRAIL and D269H/E195R which were produced and purified as described before (28, 30). Analytical gel filtration and non-reducing gel electrophoresis confirmed that D269H/E195R is a trimeric molecule that does not form higher degree aggregates (31). Soluble DR5 receptor, DcR1 and DcR2, and neutralizing antibodies to DcR1 and DcR2 were all from R&D systems. Other reagents were from Sigma-Aldrich unless otherwise stated.

#### MTT assay

Cell viability was measured by adding 500  $\mu$ g/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to control and treated cells, and incubated for 3 h at 37°C. The reaction was stopped and the purple formazan precipitate formed was dissolved in 10% dimethyl formamide and 20% sodium dodecyl sulfate and the color intensity was measured at 550 nm using a Wallac multilabel counter (32). The control value corresponding to untreated cells was taken as 100% and the viability of treated samples was expressed as a percentage of the control.

#### Annexin V staining

Externalization of phosphatidyl serine (PS) on the plasma membrane of apoptotic cells was detected using Annexin V-FITC (IQ Corporation, Groningen, The Netherlands) and flow cytometry (FacsCanto II flow cytometer, Beckton Dickinson) as previously described (33).

#### Western blotting

Polyacrylamide gel electrophoresis and Western blotting was carried out as previously described (34). For antigen detection membranes were incubated with antibodies to actin (1:500; Sigma), caspase-3 and -8 (1:1,000; Cell Signaling Technologies) overnight at 4°C followed by 2 h incubation at room temperature with appropriate secondary antibodies (1:5,000, Pierce). Protein bands were visualized using Supersignal Ultra Chemiluminescent Substrate (Pierce) on X-ray film (Agfa).

#### **Detection of caspase activity**

Cells  $(3x10^5)$  were resuspended in PBS  $(25 \ \mu$ l) and were transferred to a microtitre plate and snapfrozen over liquid nitrogen. To initiate the reaction, 50  $\mu$ M of the caspase substrate DEVD-AMC (carbobenzoxy-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin) (Peptide Institute Inc., Osaka Japan) in assay buffer (100 mM Hepes buffer, 10% sucrose, 0.1% 3[(3cholamidopropyl)dimethylammonio]-1 propanesulphonate (CHAPS), 5 mM DTT and 0.0001% Igepal 630, pH 7.25) was added to cell lysates. Substrate cleavage leading to the release of free AMC was monitored at  $37^{\circ}$ C at 60 sec intervals for 25 cycles using a Wallac multilabel counter (excitation 355 nm, emission 460 nm). Enzyme activity was expressed as nmol AMC released/min/mg protein.

#### **SPR Receptor Binding Assay**

Binding experiments were performed as previously described (28).

#### **Cell surface expression of TRAIL receptors**

Cells were washed twice in PBS containing 1% BSA and then incubated with monoclonal antibodies to DR4, DR5, DcR1 or DcR2 (Enzo Life Sciences) for 40 min on ice. The antibodies have no cross-reactivity amongst the four TRAIL receptors (shown by the Supplier's data sheet). After two wash steps with PBS/BSA, anti-mouse IgG-FITC (Sigma) secondary antibody was added for 30 min. All incubations were carried out on ice. Negative controls contained isotype control antibody. Receptor expression was analyzed on a FacsCalibur or FacsCanto II flow cytometer (Beckton Dickinson).

#### Mathematical modeling of ligand-receptor interaction

Receptor binding by WT TRAIL and D269H/E195R on the cell surface was simulated using a mathematical model describing all possible binding events. Both the formation of homotrimeric (e.g. TRAIL-3DR5) and heterotrimeric ligand-receptor complexes (e.g. TRAIL-2DR5-DcR2) were allowed and binding was simulated in a stepwise fashion (Figure 1). On-rates and off-rates measured for interactions between monomeric DR4, DR5, DcR1 and DcR2 receptors and of TRAIL binding to these receptors were taken from the report of Lee and colleagues where monomeric TRAIL-receptor constructs have been employed (25). This allowed us to derive stepwise binding constants and to model heteromeric receptor-ligand interactions (Figure 1, Supplementary Table 2 A and B). Stepwise constants for going from a single ligand-bound receptor, via two ligand-bound receptors, to the complex consisting of 3 receptors bound by a trimeric ligand were estimated in the following way: the  $k_{on}$  reported by Lee *et al.*, was assigned to the first binding event. The first association step is entropically the most unfavorable one due to loss of rotational degrees of freedom when going from an unbound state to a bound state. To compensate for this entropic penalty in the first step, the K<sub>d</sub> for the second and third step was decreased by 2 kcal/mol and

attributed completely to an increase in  $k_{on}$  (35, 36). Values of 0 to -4 kcal/mol for the entropy penalty were tested; the impact on the final results was negligible. The reported  $k_{off}$  was assigned to the first step. Two scenarios were explored: 1) no cooperative binding, i.e. binding of one receptor does not enhance the binding of the next receptor (Suppl. Table 2A) and 2) cooperative binding, whereby binding to the first receptor enhances the binding of a second receptor of the same type (Suppl. Table 2B). Co-operativity can be the result of interaction between the intracellular death domains of the DR4 or DR5 receptors, as it has been shown for FAS, another death receptor (37). Thus, to model co-operativity, the koff was decreased stepwise five fold in each subsequent binding reaction (38, 39). Varying this factor between 2 and 10 revealed little impact on the final results. In case of homotrimeric TRAIL-3DR5 and TRAIL-3DR4 complexes, an extra step to an "activated" receptor complex was introduced in order to mathematically describe the assembly of the intracellular death-inducing signaling complex (DISC, consisting of the death receptors, FADD and caspase-8). As mentioned before, DcR1 does not contain an intracellular domain and DcR2 only contains a truncated Death domain, therefore no "activation" step was added for decoy receptor containing complexes (Supplementary Table 2 A and B). The rate constants for the "activation" step that governs the formation of activated homotrimeric death receptor complexes were obtained by increasing the  $k_{on}$  and decreasing the  $k_{off}$  by a factor of ten relative to the rate constants of the third step (supplementary Table 2A and B). The presence of an activation step was necessary for scenario 1 (no cooperative effects upon binding additional receptor units) to drive TRAIL-3DR4/TRAIL-3DR5 complex formation, but the effect of the actual magnitude of this step was low upon varying this factor between 2 to 10 (relative to the rate constants of the third step). The impact on inclusion of an activation step for scenario 2-the co-operative model-was relatively low.

Reaction equations describing the binding of TRAIL to preassembled dimeric receptor complexes

was modeled in the following way: the  $k_{on}$  of the first step  $(k_{on}^{A})$  and the  $k_{off}$  of the second step  $(k_{off}^{B})$  were attributed to binding equations describing free TRAIL binding a preassembled receptor complex and, in case of equations describing the binding of single bound TRAIL (i.e. TRAIL-DR5) to a preassembled receptor complex, the  $k_{on}$  of the second step  $(k_{on}^{B})$  and the  $k_{off}$  of the third step  $(k_{off}^{C})$  were attributed to the reaction. When the pre-assembled receptor complex was a heterodimer (i.e. DR5-DR4) the rate constants of the receptor with the rate-limiting kinetics, i.e. the receptor having the slowest  $k_{on}$  and the fastest  $k_{off}$ , were chosen.

The number of DR5 receptors on the surface of Colo205 was estimated by comparing the receptor expression level on the surface of Colo205 to MD MBA-231 cells and subsequently relating this ratio to the number of surface expressed receptors determined for MD MBA-231 cells (40) (Suppl. Fig 1A and B; Suppl. Table 1). The relative expression of the four TRAIL receptors at the single cell level has also been estimated by simultaneously labeling all four receptors using four separate fluorochromes for the four anti-TRAIL receptor antibodies and analysis of the fluorescence intensity on 10,000 cells by flow cytometry. By selecting the cells expressing very similar levels of one receptor, the expression of the other three receptors on the same subset of cells was analyzed. Apparent affinities for D269H/E195R were taken from van der Sloot *et al.*, (28). The increase in affinity of D269H/E195R for DR5 was completely attributed to the kon and determined to be 3 to 5 fold, as changes in off-rate in the SPR sensorgram between WT TRAIL and D269H/E195R could not be calculated due to minimal and very slow rate of ligand dissociation. The decrease in affinity of D269H/E195R for the other receptors was equally attributed to the  $k_{\text{on}}$  and the  $k_{\text{off}}$  based on previous studies (28). The rate constants for the equation describing the formation of the activated death receptor complexes were derived as described above for WT.

To mimic the local concentration of the receptors at the cell membrane, a volume equal to sphere of 10 nm around a typical mammalian cell was taken as the simulation volume (13.9  $\mu$ m<sup>3</sup>). The initial

steady state distribution of the receptors between monomeric, homo- and hetero-dimeric species was obtained by running the model with the TRAIL concentration set at 0. Unless otherwise specified, all simulations were performed at a TRAIL concentration of 25 ng/ml ( $\sim$ 1.3x10<sup>-9</sup> M). The model was simulated using the stochastic *next subvolume* method (41, 42) as implemented in SmartCell version 4.3 (27, 43). SmartCell is available at http://software.crg.es/smartcell.

#### Results

#### Mathematical modeling of ligand-receptor interaction

To investigate the hypothesis that the kinetics of receptor activation of promiscuous ligand-receptor systems is governed by non-productive heteromeric interactions and that the kinetics of such systems could be modified by changing specificity, we simulated the TRAIL ligand-receptor interaction occurring on the cell surface using a mathematical model. The model was set up to simulate the interactions occurring on the membrane of Colo205 cells upon exposure of the cells to WT TRAIL or the DR5-selective variant, D269H/E195R (28) (Figure 1). TRAIL mediates cell death in Colo205 cells by directly activating the downstream caspase members of the protease cascade (type I apoptosis)(11) and does not require activation of the intrinsic apoptotic pathway (Suppl Fig. 2).

The ratio of DR4, DR5, DcR1 and DcR2 was set to reflect the true ratio of the four receptors on the surface of Colo205 cells as described in Materials and Methods (Suppl. fig. 1A and B; Suppl. Table 1) and the ratio of TRAIL receptor expression has been estimated at single cell level by simultaneous fluorescent (multicolor) labeling the four TRAIL receptors (Suppl. fig. 1C). Subpopulations of cells that expressed very similar amount of one receptor showed a much wider distribution for the expression of the other three receptors. Nonetheless, there was a proportional relationship in TRAIL receptor expression; higher expression of one receptor correlated with a higher average expression of the other three receptors (cross comparison of DR4 expression to DR5, DcR1, DcR2 is shown in Suppl. Fig 1C, cross-comparison of all receptor pairs have been carried out with similar results). We used, when it was possible, experimental binding values for WT TRAIL and the DR5-specific mutant (25, 28) or reasonable estimates (as detailed above). The model was chosen to simulate the binding of the ligand (WT TRAIL or D269H/E195R) to the receptors in a stepwise process and to allow for the formation of both homotrimeric (e.g. TRAIL-

3DR5) and heterotrimeric receptor complexes (e.g. TRAIL-2DR5-DcR2). Key assumptions of the model were: A) Pre-ligand assembly of receptors result in homo- and heterodimeric receptor complexes (44, 45). B) Formation of the active homotrimeric receptor complex after TRAIL binding stabilizes the complex (only for DR5 and DR4 receptors; DcR1 and DcR2 have no cytoplasmic domain or a truncated cytoplasmic domain, respectively, that can oligomerize; see Methods) and heterotrimeric receptor-TRAIL complexes could exchange receptor subunits as it has been shown for other members of the receptor family, TNF-R1 and TNF-R2 (46). C) Heterotrimeric receptor-TRAIL complexes do not induce apoptosis (20, 24, 47). Two different scenarios were explored: 1) No explicit cooperativity/avidity effects are present between receptor monomers upon stepwise binding of TRAIL, i.e.  $k_{off}^{A} = k_{off}^{B} = k_{off}^{C}$  (Suppl. Table 2A) and 2) Cooperative/avidity effects present between receptors of the same species, e.g. due to interactions between membrane proximal parts of the receptors or its intracellular domains (37), which was modeled by decreasing the  $k_{off}$  stepwise:  $k_{off}^{B} = 1/5k_{off}^{A}$  and  $k_{off}^{C} = 1/5k_{off}^{B}$  (see Methods; Suppl. Table 2B). Of note, this latter situation does not refer to the PLAD-PLAD interactions that are present between the receptors in the unbound state as no such interactions have been observed in crystal structures of TRAIL in complex with DR5(48, 49). Unless otherwise specified all results refer to scenario 1: no explicit avidity/cooperativity effects present. A model that did not include heteromeric interactions and only allowed the formation of homomeric TRAIL-receptor complexes was constructed as an additional control.

When the binding of WT TRAIL to the four TRAIL receptors was simulated, the  $1/2T_{max}$  (i.e. time when 50% of the final response was reached) of the WT TRAIL-3DR5 complex was reached within ~1100 s, while the  $1/2T_{max}$  for DR4 was reached after ~2300 s (Fig. 2A). The number of WT TRAIL-3DR5 complexes formed after 1 h was the highest, while the number of TRAIL-3DR4,

TRAIL-3DcR1 and TRAIL-3DcR2 were 90%, 35% and 1% of the number of TRAIL-3DR5 complexes, respectively. In contrast, the  $1/2T_{max}$  for D269H/E195R-3DR5 complex formation was reached after 130 s and the amounts of D269H/E195R-3DR4, -3DcR1 or -3DcR2 complexes were much lower even after more than 1 h of simulation (45%, 1% and 5%, respectively) (Fig. 2B). The total number of D269H/E195R-3DR5 complexes was ~15% higher than of WT TRAIL-3DR5 by the end of the simulation.

The slightly higher affinity and the 3-5 fold higher receptor activation kinetics of D269H/E195R towards DR5 cannot explain the predicted, 10 fold increase in receptor activation as a theoretical TRAIL (Control-1) variant only showed a modest improvement in TRAIL-3DR5 activation rate (Fig 2C and 2D). This variant has the rate constants of D269H/E195R for DR5, but rate constants for the other receptor are the ones of WT TRAIL, and it mimics a situation of an affinity increase for DR5 but without a concurrent large change in receptor specificity. On the other hand, when the ligand (theoretical TRAIL variant Control-2) was attributed the rate constants of WT TRAIL for DR5 and the rate constants of D269H/E195R for DR5, resulted in a significant enhancement in the rate of DR5 activation (Fig. 2C and 2D). A third control (Control-3) mimics a situation where the affinity increase is equivalent for all receptors (modeled by increasing all WT k<sub>on</sub> rates fivefold) and shows only a very modest improvement in TRAIL-3DR5 activation rate (Fig. 2C and 2D).

Thus, the rate of receptor activation can be enhanced by simply increasing the specificity towards a target receptor without changing the binding kinetics toward this target receptor. Performing all simulations under condition 2 (by including a co-operativity effect) revealed the same trends (Suppl. Fig. 3). In contrast, kinetics of receptor activation by WT TRAIL would be substantially

faster when only homo-oligomerization of receptors would occur. The mathematical model that allows only homo-oligomerization reactions showed that the  $1/2T_{max}$  of WT TRAIL and of D269H/E195R was already reached after 670 and 140 seconds, respectively (Suppl. Figure 4). Thus, under these conditions the speed of DR5 receptor activation of D269H/E195R is not affected while the activation speed of WT TRAIL is substantially enhanced and the difference between the two ligands can then solely be explained by the difference in k<sub>on</sub> for the DR5 receptor. Hence, according to the heteromeric interaction model, it is therefore the decreased affinity for DR4, DcR1 and DcR2 (i.e. its increased specificity towards DR5), in addition to the increase in association onrate for DR5 of the mutant that changes the speed of receptor activation (Fig 2C and 2D).

To analyze the sensitivity of the model to changes in parameters the simulations were repeated by varying WT/ D269H/E195R concentrations, the number of decoy receptors per cell or using different combinations of  $k_{on}/k_{off}$  rate constants while keeping the K<sub>d</sub> constant (using the relationship K<sub>d</sub>= $k_{off}/k_{on}$ ). This analysis shows that the speed of receptor activation remains dependant on receptor specificity (Supplementary figure 5). Analyzing the 1/2T<sub>max</sub> of TRAIL-3DR5 complex formation reveals that the activation rate of DR5 by WT TRAIL is more buffered against changes in concentration than D269H/E195R (Suppl. fig. 6A). In contrast, the activation of DR5 by WT TRAIL is more buffered against changes in concentration than D269H/E195R (Suppl. fig. 6A). In contrast, the activation of DR5 by WT TRAIL is more buffered against changes in the number of decoy receptors than D269H/E195R (Suppl. fig. 6B). The response to changes in  $k_{on}$  /  $k_{off}$  combinations remains similar for WT TRAIL and D269H/E195R (Suppl. fig 6C). In this latter case the relative specificity remains the same under varying  $k_{on}$  /  $k_{off}$  combinations thereby explaining a similar behavior for WT and D269H/E195R. In contrast, upon changing the number of decoy receptors the promiscuous behavior of WT TRAIL is much more affected (either augmented or attenuated upon an increase or decrease in the number of decoy receptors, respectively) than D269H/E195R, resulting in a more

sensitive behavior of WT TRAIL. The reverse is the case upon changing the ligand concentration, while the response to an increase in WT TRAIL concentration is attenuated due to interactions with all four receptors, a similar increase in D269H/E195R concentration is more exclusively available to bind the DR5 receptor, hence resulting in a more sensitive behavior of D269H/E195R upon changes in ligand concentration.

As a summary the change in receptor activation kinetics can be explained by the fact that WT TRAIL triggered the formation of a much higher degree of heterotrimeric receptor complexes than D269H/E195R, especially during the first 30 min of incubation (Supplementary figure 7). This receptor pool dynamically changed and gradually disappeared over the course of incubation, due to rearrangement into active homotrimeric complexes, resulting in a slower activation of caspase-8 and apoptotic signaling.

#### DR5-selective variants activate DR5 at a faster rate than WT TRAIL

To validate the predictions of the model, Colo205 colon carcinoma cells were incubated with WT TRAIL or D269H/E195R for varying times (5-180 min), after which the ligands were washed out and the incubation continued in normal growth medium for 180 min in total. The kinetics of death receptor activation by the two ligands was monitored by measuring death receptor-mediated procaspase-8 activation and induction of cell death. While D269H/E195R only required  $3.6 \pm 0.4$  min to reach 50% of its total efficacy (which was  $87.2 \pm 3.9\%$  Annexin V positivity), WT TRAIL required  $60.8 \pm 4.3$  min incubation with the cells to reach the same level (i.e. 50% of its total efficacy of  $80.8 \pm 5.4\%$  Annexin V positive dead cells), showing that receptor activation by D269H/E195R occurs 17 times faster than with WT TRAIL (Fig. 3A). The faster kinetics of apoptosis induction correlates with a faster rate of receptor activation as monitored by activation of

caspase-8: D269H/E195R induced pro-caspase-8 processing into its active form within 5 min incubation, while 60 min was required for a comparable level of pro-caspase-8 processing by WT TRAIL (Fig. 3B). This increase of receptor activation of D269H/E195R and subsequent apoptosis induction is in good agreement with the predictions of our model (Figure 2A and B). Taken together, these data suggest that the degree of TRAIL dependent receptor hetero-oligomerization regulates the kinetics of TRAIL induced apoptosis.

#### Decoy receptors slow down the kinetics of death receptor activation

Another prediction of the model suggests that preventing binding to the decoy receptors should increase the kinetics of receptor activation and thus induction of apoptosis by WT TRAIL but not of the DR5-specific mutant. Simulating WT TRAIL or D269H/E195R receptor binding without decoy receptors present showed that DR5 activation occurs 1.5 fold faster for WT TRAIL, whereas the activation of DR5 by D269H/E195R was not affected (Figure 4A and B). To confirm this prediction, Colo205 cells were incubated with neutralizing antibodies to DcR1 or DcR2 for 1 h prior to treatment with the EC<sub>50</sub> dose of WT TRAIL (20 ng/ml) or D269H/E195R (4 ng/ml) and induction of apoptosis was measured 3 h post-treatment using Annexin V assay (Fig. 4C). The enhancement of apoptosis in response to decoy receptor neutralization was calculated as fold increase compared to the level of apoptosis induced by the ligands in the absence of the neutralizing antibodies. Neutralization of DcR1 and DcR2 resulted in a significant,  $1.51 \pm 0.15$  and a  $1.22 \pm 0.07$ fold increase in WT TRAIL-induced apoptosis, respectively. Combining the neutralizing antibodies to DcR1 and DcR2 had an additive effect ( $1.68 \pm 0.12$  fold increase of percentage apoptosis, Fig. 4C). On the other hand, decoy receptor neutralization did not enhance apoptosis-induction by D269H/E195R treatment, indicating that the decoy receptors cannot counteract death receptor activation by D269H/E195R, only by WT TRAIL. Inhibition of the decoy receptors also increased

the kinetics of death receptor-activation. Pre-treatment of the cells with decoy receptor neutralizing antibodies significantly accelerated WT TRAIL-, but not D269H/E195R-induced accumulation of Annexin V positive cells, by  $1.5 \pm 0.1$  fold (Fig. 4D, 4E), as well as TRAIL-mediated pro-caspase-8 processing (Fig 4F).

#### DR5-selective TRAIL variants are more effective in tumor cell killing

If the kinetics of receptor activation regulates apoptosis signaling, D269H/E195R should have a higher tumor cell killing efficacy than WT TRAIL at lower concentrations and within a shorter time frame. Treatment of the cells with 2-30 ng/ml of the DR5-selective variant, D269H/E195R for 3 h induced a 3.3-4.2 fold higher cytotoxicity than WT TRAIL as determined by Annexin V assay (Fig. 5A). Activation of the downstream executioner caspases (caspase-3 and -7, determined by a DEVDase assay) also showed that a 2.9-4.1 fold lower concentration of the DR5-selective variant was sufficient to induce the same level of apoptosis as WT TRAIL (Fig. 5B). The higher DEVDase activity induced by the DR5-selective mutant after 3 h treatment correlated with stronger procaspase-8 processing detected by Western blotting (Fig. 5C). These results underline our prediction that fast death receptor activation results in the simultaneous activation of a large number of procaspase-8 molecules, which in turn ensures that the apoptotic signal spreads throughout the cells and the tumor cell commits to death.

# D269H/E195R used in combination with aspirin can induce enhanced synergism compared to WT TRAIL.

Various chemotherapeutics and irradiation have been reported to sensitize tumor cells to TRAIL, which correlated with induction of DR4 and/or DR5 (50). Many of these treatments however, also induce the expression of the decoy receptors that can limit the efficacy of WT TRAIL (51). For

example, aspirin (2.5-5 mM for 24 h) induced the expression of DcR1 and DcR2 alongside with DR5 on the surface of Colo205 cells (Fig. 5D). When aspirin at a dose that induced minimal loss of viability in Colo205 cells (2.5 mM) was combined with WT TRAIL, it enhanced WT TRAILinduced effector caspase activity and loss of cell viability at every timepoint studied (p < 0.05 at every timepoint where WT TRAIL was added, determined by paired student t-test, Fig. 5E and Suppl. Fig. 8). However, when WT TRAIL was replaced with D269H/E195R in the treatment, the potentiating effect, reflected in the level of effector caspase activation, was much more pronounced. with a significantly higher DEVDase activity in the D269H/E195R+aspirin samples that in the WT TRAIL+aspirin samples after 2 h and 3 h treatments (p < 0.05, Fig. 5E). Since disintegrating dead cells lose their cellular content including caspases, the accumulative effect of the combination treatment was monitored using a viability (MTT) assay (Suppl. Fig. 8). These results show that induction of cell death was not only faster by D269H/E195R as seen in the DEVDase assays, but also reached a higher maximum level. The combination index (CI) was calculated for the two treatment combinations (aspirin with WT TRAIL vs. aspirin with D269H/E195R) using the Chou-Talalay method(52) and it clearly showed that the potentiating effect with D269H/E195R was much larger than with WT TRAIL and across a wider effect-level range (Fa values: reduction in viability on a scale of 0.01-0.99 with 1 being the untreated control, Suppl. Fig. 8). This confirms that enforcing fast, homotrimeric DR5 complex formation using a receptor-selective ligand that bypasses the formation of inhibitory TRAIL-heteromeric receptor complexes is central to effective apoptosis induction and thus tumor cell killing.

#### Discussion

The outcome of signaling events of ligand-receptor systems that consist of multiple receptors and/or ligands is complex; such multi-component systems can yield different biological responses and the individual components can add different layers of regulation. Because these systems enable diverse biological responses, they are very common. Examples include the vascular endothelial growth factor ligand-receptor system, the fibroblast growth factor or the TRAIL ligand-receptor systems (2, 4, 8).

Extrapolation of kinetic and equilibrium constants from *in vitro* measurements of purified recombinant ligands and receptors to *in vivo* conditions is challenging when dealing with such complex cases as the molecule that triggers the signal can be trapped by non-productive interactions that equilibrate slowly with the productive complex. This is not only the case with the aforementioned multiple receptor/ligand systems but in principle also extends to promiscuous intracellular adaptor proteins that can be trapped in non-productive interactions. Not accounting for these non-productive interactions in signal transduction simulations can result in overestimating of (activation) kinetics and, subsequently, result in the wrong interpretation of the underlying signaling networks and the biological response.

We hypothesized that the kinetics of receptor activation is a major determinant in signal transduction and that formation of non-productive interactions can severely change the observed activation kinetics and even the final biological response. As a model, we choose one such multi-component system, TRAIL and its cognate receptors, and examined how apoptosis signaling by the different TRAIL receptors is regulated. Tight regulation of apoptosis signaling is crucial for the survival of multi-cellular organisms and deregulation of apoptosis culminates in various diseases

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(e.g. cancers, neurodegenerative diseases and autoimmune diseases) (53). Regulation of apoptosis is provided at several levels in the apoptosis signaling cascade (both upstream, at the level of the receptor and at various downstream levels). With the aid of a previously developed DR5-selective TRAIL variant and mathematical modeling, we investigated the regulation provided by the presence of multiple TRAIL binding receptors and their competition for the ligand.

Wild type TRAIL can bind to its death receptors and decoy receptors with a broadly similar degree of affinity (54, 55). The DR5-selective TRAIL variant has a 3-5 fold increase in DR5 binding kinetics combined with a many fold decrease in binding kinetics to DR4 and DcR1 and a modest decrease in binding kinetics to DcR2. At first sight, common sense would likely predict a 3-5 fold increase in the kinetics of apoptosis induction for this DR5 selective variant. But our studies, qualitatively in line with the predictions of our simulation model, revealed that the DR5-selective TRAIL variant both activates the death-inducing DR5 receptor and induces apoptosis at approximately 20 times faster than WT TRAIL. In a previous study we have demonstrated the same, i.e. faster receptor-activation kinetics, using a DR4-selective TRAIL variant (7).

The simulation model we have generated showed that the higher the specificity of the ligand towards the target receptor the faster it will activate that receptor. Furthermore, initiation of the signal transduction pathway is regulated kinetically by the non-signaling TRAIL receptor complexes (e.g. TRAIL-2DR5-DcR2 or TRAIL-DR5-2DcR2). Low degree of receptor specificity of the ligand leads to the transient formation of heteromeric receptor complexes that gradually rearrange into functional, homotrimeric complexes. In case of WT TRAIL we have found that it results in a slow activation of the death-inducing receptors and cleavage of pro-caspase-8. This does not only slow down the initiation of the signal, but can completely abrogate it. Cleaved caspase-8,

especially the catalytically active p18 fragment, has been shown to have a short half-life time of approximately 20 min as it is targeted for proteasomal degradation by Caspase-8 and -10 associated RING proteins 1 and 2 (CARP-1, -2) (15, 17). Slow and inefficient pro-caspase-8 activation is thus insufficient to induce apoptosis and this is one of the reasons tumors can be sensitized towards TRAIL by proteasome inhibitors (15, 56, 57). A ligand with high specificity towards one receptor can avoid this kinetic hindrance driven by non-signaling heteromeric complexes and trigger much faster receptor activation thereby producing high amounts of activated caspase-8 molecules in a short time, resulting in fast and robust induction of apoptosis. Using the DR5 selective TRAIL variant or WT TRAIL ligand in the presence of neutralizing antibodies against the non-signaling TRAIL-receptors, we have provided evidence that this hypothesis is true.

Tight regulation of apoptosis signaling is crucial for the survival of multi-cellular organisms and loss of this regulation contributes to serious diseases (e.g. cancers, autoimmune diseases, neurodegenerative disorders (53)). For example, spurious or low level caspase activation that is insufficient to trigger a unified apoptotic response of the cell, but could potentially cause local damage by inducing intermediate states of partial cell death, is actively removed by proteolysis (16). In line with our results, analysis of the kinetics of apoptosis at a single cell level showed that effector caspase activation is an all or nothing event and is independent of the dosage of the apoptosis-inducing stimulus (58). On the other hand, pro-caspase-8 activation was found to occur in a dose dependent manner in response to TRAIL (59). These results insinuate that the commitment to apoptosis is most likely occur at the level immediately downstream of the receptor (14, 60). Until caspase-8 activity reaches a certain threshold, the death signal is not transmitted to the execution machinery and thus guarantees the uniform "all-or-nothing" response of the cell upon commitment to death (14, 60).

It is well established that overexpression of the decoy receptors inhibits TRAIL-induced apoptosis (19, 51). However, the potency of the decoy receptors expressed at endogenous levels to antagonize TRAIL-induced apoptosis and thus their real physiological function is largely unknown. Previous studies also found that in some tumor cells the expression of the decoy receptors did not correlate with TRAIL-sensitivity (61-63). This could be due to inhibitors of apoptosis acting downstream of the receptor (e.g., c-FLIP, Bcl-2, XIAP) or compartmentalization of the receptors (64, 65), but the exact mechanism of decoy receptor-mediated TRAIL antagonism is not fully understood yet. Apart from sequestering available TRAIL (25), DcR1 and DcR2 can also interfere with the formation of functional, homotrimeric DR4 and DR5 signaling complexes. Clancy and colleagues reported that DcR2 can bind to DR5 in a ligand (TRAIL)-independent manner via PLAD-PLAD domain interactions of the receptors, which inhibits the formation of functional TRAIL-DR5 signaling complexes (24). On the other hand, a study by Merino et al., showed that TRAIL can co-recruit DcR2 with DR5 producing a heteromeric, inactive receptor complex, thus equally inhibiting DR5 function but in a ligand-dependent manner (20). Our results suggest that rather than simply sequestering available TRAIL, decoy receptors might provide a kinetically driven buffer against unwarranted or spurious caspase 8 activation by low concentrations of TRAIL by forming inactive heteromeric receptor complexes in a ligand dependant way and thereby reduce noise in TRAIL induced apoptosis signaling.

In conclusion we can state that receptors of a multi-receptor system can work in concert to create a temporal control of signal transduction. Our results indicate that signal transduction induced by promiscuous ligands is largely determined by non-productive interactions and consequently, by the relative amount of the different receptors sharing the same ligand. The observation that binding of a ligand to multiple types of receptor can slow down the activation of the preferred target receptor has

also implications at a more applied level. It can be used to increase the efficacy of promiscuous cytokines to a particular receptor with the aim to develop more potent therapeutics. Faster kinetics can be achieved by increasing the  $k_{on}$  for the target receptor and by simultaneously decreasing the affinity for the other receptors, i.e. both the relative and the absolute affinity for the target receptor must increase to generate fast acting and thus strong agonists/antagonists. For example, in case of WT TRAIL, considering its short *in vivo* half-life time (66), faster receptor binding kinetics (and faster induction of apoptosis) can generate a considerable therapeutic advantage.

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#### **Figure legends**

Figure 1. Schematic of stepwise ligand-induced TRAIL receptor oligomerization used to model the kinetics of *in vivo* TRAIL receptor activation. Receptor ligation by TRAIL was modeled by describing all possible binding events. Both the formation of homotrimeric (e.g. TRAIL-3DR5) and heterotrimeric ligand-receptor complexes (e.g. TRAIL-2DR5-DcR2) were allowed and binding was simulated in a stepwise fashion. (A) Stepwise model of ligand-induced TRAIL receptor trimerization. The receptors could either be the death-inducing (DR4, DR5) or decoy receptors (DcR1, DcR2) indicated by the dashed outline of the death domain. (B) Active and inactive receptor complexes. In case of homotrimeric TRAIL-3DR5 and TRAIL-3DR4 complexes (left hand unit), an extra step to an "activated" receptor complex was introduced in order to mathematically describe the assembly of the intracellular death-inducing signaling complex (DISC, consisting of the death receptors, FADD and caspase-8).

**Figure 2.** Mathematical modeling of TRAIL receptor complex formation triggered by WT TRAIL and D269H/E195R over time. (A) Formation of ligand-bound, homotrimeric DR4, DR5, DcR1 and DcR2 complexes after exposure to WT TRAIL (WT) or (B) D269H/E195R (DE). (C) Homotrimeric DR4 and (D) DR5 formation by theoretical TRAIL variants 1, 2 and 3 (control 1, control 2 and control 3). Control 1 variant binds to DR5 with the affinity of D269H/E195R and with the affinity of WT TRAIL to the other three TRAIL receptors. Control 2 binds to DR5 with the same affinity as WT TRAIL, but has the same affinity as D269H/E195R towards DR4, DcR1 and DcR2. Control 3 variant binds to DR5 with 5 fold higher affinity (similar to D269H/E195R) and shows a concurrent equivalent change in affinity towards DR4, DcR1 and DcR2.

Figure 3. D269H/E195R activates death-inducing TRAIL receptors and apoptosis an order of magnitude faster than WT TRAIL. Colo205 cells were treated with 30 ng/ml of WT TRAIL or D269H/E195R for the times indicated, after which the ligands were washed out and the cells were incubated in normal growth medium for 180 min (for TRAIL receptor activation) or for 240 min (for phosphatidylserine externalization) in total. (A) Kinetics of apoptosis induction by WT TRAIL and D269H/E195R measured by Annexin V assay. Apoptosis induction was expressed relative to the percentage of death induced after 240 min without washing out the ligands  $\pm$  S.E.M from three independent experiments. The percentage of Annexin V positive cells induced by D269H/E195R was significantly higher at every timepoint than that of induced by WT TRAIL (paired student t-test,  $p \le 0.05$ ). The data was fitted with an interpolating spline using the software MatlabR from which the time to induce 50% efficacy was determined. (B) Kinetics of death-inducing TRAIL receptor activation by WT TRAIL and D269H/E195R was measured by WT TRAIL and D269H/E195R was measured by annexin V assister blotting. The levels of actin are presented as loading controls. The images are representatives of three independent experiments.

Figure 4. Decoy receptors reduce TRAIL-induced apoptosis and kinetics of DR4/DR5 activation. Mathematical modeling of DR5 and DR4 receptor activation in the presence (+) or absence (-) of decoy receptors of (A) WT TRAIL or (B) D269H/E195R. Experimental validation: (C) DcR1 and DcR2 neutralization augments WT TRAIL- but not D269H/E195R-induced apoptosis. Colo205 cells were incubated for 1 h with 2  $\mu$ g/ml of neutralizing antibodies to DcR1 and DcR2, followed by treatment with WT TRAIL (20 ng/ml) and D269H/E195R (4 ng/ml) for 3 h. The graph shows fold increase in Annexin V binding calculated by dividing the percentage apoptosis induced by the ligands in the presence of the antibodies ±S.E.M. \* indicates

significant differences determined by paired t-test (p<0.01) based on results from three independent experiments. (D) Effect of decoy receptor neutralization on the kinetics of receptor activation by WT TRAIL and (E) for D269H/E195R. Colo205 cells were treated as described at point C., At the indicated time points the ligands were washed out and the cells were incubated in normal growth medium for 240 min in total. Apoptosis induction was measured by quantifying phosphatidyl serine exposure and expressed relative to the percentage of death induced after 240 min without washing the ligands out. The graphs show the averaged result from three independent experiments  $\pm$  S.E.M. The percentage of Annexin V positive cells induced in the presence of DcR1/2 neutralizing antibodies was significantly higher at every timepoint than that of induced by WT TRAIL alone (paired student t-test,  $p \le 0.05$ ). (F) Neutralization of the decoy receptors enhances TRAIL-mediated pro-caspase-8 processing. Colo205 cells were treated with WT TRAIL +/- DcR1/2 neutralizing antibodies as described above in points C, D for the analysis of pro-caspase-8 processing by Western blotting. The figure shows pro-caspase-8 processing induced by WT TRAIL alone, or after inhibition of the decoy receptors in alternating lanes. The image is a representative of samples pooled together from two independent experiments. The level of actin is shown for loading control.

Figure 5. DR5-selective variants are more efficient in killing DR5-sensitive tumor cells than WT TRAIL. Colo205 cells were treated with WT TRAIL or DR5-variant (5-30 ng/ml) and induction of apoptosis was monitored. (A) Phosphatidylserine externalization. The graph shows averaged percentage apoptosis induced  $\pm$  S.E.M. (B) Caspase activity (DEVDase), measured in a kinetic assay. Enzyme activity expressed in nmole AMC released per min by 1 mg of total cellular protein. (C) Western blot analysis of pro-caspase-8 cleavage (activation) induced by WT TRAIL or D269H/E195R. D269H/E195R used in combination with aspirin can induce enhanced synergism compared to WT TRAIL. (D) Aspirin induces expression of DR5, DcR1 and DcR2 on the cell surface. Colo205 cells were treated with 5 mM aspirin for 24 h and cell surface expression of DR4, DR5, DcR1 and DcR2 was measured by immunostaining and detected with flow cytometry. The histograms shown include isotype (negative) control (black, open peak), untreated Colo205 (labeled: Cont; grey, closed peak) and aspirin-treated Colo205 (labeled: Asp; grey, open peak) samples. The histograms are representatives of three independent experiments. (E) Caspase activation by combined treatment with aspirin plus WT TRAIL or D269H/E195R. Cells were treated with 5 mM aspirin for 24 h followed by 5 ng/ml of WT TRAIL or D269H/E195R for the times indicated, after which the cells were harvested and DEVDase activity as a measure of cell death was measured in cell lysates. DEVDase activity was expressed as average nmole AMC released per min by 1 mg total cellular protein. The results are mean values  $\pm$  SD of 3 independent experiments. \*, \*\* indicate significant differences between the connected sample pairs (p < 0.05).

Fig. 1. Szegezdi e.a. TRAIL-induced receptor trimerization



Homo- and heteromeric complex formation





inactive







A







Fig. 4. Szegezdi et al.



А





E195R

DR5

← Cont