#### <u>Chapter 5</u>

# 5. Objective III: Study the effect of TRAIL on theophylline mediated cell death and/or mitotic arrest.

#### 5.1. Background study

In the previous objectives we have addressed questions related to recombinant TRAIL treatment and TRAIL resistance for understanding and developing better ways to treat breast cancer with TRAIL, next we come to combination therapy as it has been proven effective in sensitized cells towards TRAIL treatment. We identified a drug called theophylline, to be used in combination with TRAIL, as previous studies have already shown theophylline to be toxic to TMBC cell line<sup>166</sup> and this drug is already present in the market for treating respiratory disorders.

Theophylline, also known as 1,3-dimethylxanthine, is a type of methylxanthine drug used for treating respiratory diseases for more than 80 years. It naturally occurs in tea and cocoa beans in trace amounts. It was initially extracted from tea. For the first time in 1895, theophylline was chemically synthesised and used as a diuretic<sup>167</sup>. Theophylline is known to be involved in inhibition of phosphodiesterase, increase in histone deacetylase-2 activity, and upregulation of interleukine-10 secretion<sup>168</sup>. It decreases the secretion of proinflammatory cytokine IL-6 in pre-adipocytes and mouse-derived primary preadipocytes<sup>169</sup>. It is known to be an inhibitor of mitosis and has been shown to act as an anti-mitotic agent in breast cancer cells<sup>166</sup>. Theophylline has been already clinically tested one of the candidates for combination therapy against prostate cancer as (ClinicalTrials.gov NCT 01017939) and acute myelogenous leukaemia (AML) (ClinicalTrials.gov NCT00175812)<sup>170</sup>. Theophylline in combination with valproic acid and all-trans retinoic acid have shown promising results for AML patients. Theophylline have shown to increase ROS in MDA-MB-231 cell line<sup>171</sup>. Theophylline have cytotoxicity in MDA-MB-231 breast cancer cells but not in normal MCF10A breast cells<sup>168</sup>. Therefore, in our present objective, we have tried to identify the molecular mechanism behind

theophylline mediated cytotoxicity in triple negative MDA-MB-231 breast cancer cells and the effect of theophylline in combination with TRAIL.

#### 5.2. Results

#### 5.2.1. Theophylline showed cytotoxicity in breast cancer and tumorigenic human embryonic kidney cell lines but has no effect on normal fibroblast cell line

To investigate the cytotoxic ability of theophylline, we performed MTT assay after treatment with 24hrs treatment with theophylline. Cell viability assay with MDA-MB-231 breast cancer cell line showed significant reduction in cell number after treatment with theophylline (Fig. 1a). We also found similar observation of reduced cell viability with tumorigenic human embryonic kidney cell line, HEK293, after treatment with same dose of theophylline (Fig. 1b). We have also treated the 'normal' immortalized mouse embryonic fibroblast cells, NIH3T3, with theophylline but did not observe any significant difference in cell viability (Fig. 1c).



**Figure 5.1.** Theophylline decreases cell viability in tumorigenic cells but not normal cells. Cell viability was measured using MTT assay in (a) MDA-MB-231, (b) HEK293, and (c) NIH3T3 cells after treatment with theophylline for 24-hrs. All experiments were carried out in triplicate and repeated three times. Representative experiment has been shown in the figure. (C: control; T: theophylline; \* indicates  $p \le 0.05$ ).

## 5.2.2. Theophylline causes cell death, DNA damage and induction of apoptosis in MDA-MB-231 cells

We further evaluated the cytotoxic ability of theophylline via trypan blue assay. There we observed that treatment of MDA-MB-231 cells with theophylline causes significant increase in cell death in comparison to the control cells (Fig. 2a). To investigate whether theophylline treatment causes DNA damage in breast cancer cells, alkaline comet assay was performed. Theophylline treated MDA-MB-231 cells showed increased amount of total DNA damage (Fig. 2b), we even observed significant amounts of double strand DNA breaks via neutral comet assay after theophylline treatment (Fig. 2c). Acridine orange and ethidium bromide staining showed morphological changes in theophylline treated cells, which that are associated with apoptosis (Fig. 2d). There was a significant increase in early signs of apoptosis such as membrane blebbing and also increased late apoptosis was shown via presence of EtBr-stained red nucleus after theophylline treatment (Fig. 2e, 2f).



**Figure 5.2.** Theophylline showed cytotoxicity in MDA-MB-231 breast cancer cells. (a) Cytotoxicity was measured by trypan blue assay after 24-hrs of theophylline treatment. (b) Total DNA damage and (c) dsDNA damage are represented as Olive tail moment after performing alkaline and neutral comet assay after 6-hrs of theophylline treatment, respectively. (d) Representative image of cells showing membrane blebbing (arrow) after staining with AO dye. AO/EtBr staining was used to measure (e) early apoptosis and (f) late apoptosis after 6-hrs of theophylline treatment. All experiments were carried out in triplicate and repeated three times. Representative experiment has been shown in the figure. (C: control; T: theophylline; \* indicates  $p \le 0.05$ ).

#### 5.2.3. Theophylline initiates caspase-mediated apoptosis by upregulating the expression of TNFR1 receptor

Next, we investigated the initiator molecule responsible for activation of the apoptotic cascade and we found that theophylline induces apoptosis via TNFR1 death receptor. Theophylline treatment significantly upregulates the expression level of both transcript and protein in MDA-MB-231 breast cancer cells and tumorigenic HEK293 cells (Fig. 3a, 3b, 3c, 3d). We found that theophylline upregulates the entire complex of TNFR1 death receptor, in our study TRADD and cleaved RIP of the TNFR1 complex are also upregulated at the protein level after theophylline treatment in MDA-MB-231 cell line (Fig. 3e, 3f). We also found that protein level of TNFR1 ligand TNF $\alpha$  has also increased after theophylline treatment in MDA-MB-231 cells (Fig. 3g). Next, we observed that MDA-MB-231 cells treated with theophylline shows higher levels of cleaved caspase-8, -9 and -3 protein (Fig. 3h, 3i, 3j). Theophylline treated MDA-MB-231 cells also showed reduction in mitochondrial membrane potential ((Fig. 3k). To further establish the fact that TNFR1 plays an important role in activating the apoptosis pathway after theophylline treatment, we treated the cells with R7050 simultaneously with theophylline. R7050 is established antagonistic molecule against TNFR1. It acts as a blocker of TNFR1 binding to TRADD and RIP1, causing a disruption in activation of the downstream apoptotic pathway. When cells were treated in combination of R7050 and theophylline, they showed higher cell viability in comparison to the single treatment of theophylline (Fig. 31). Even the expression levels of cleaved caspase-8, -9 and -3 reduced after as compared to theophylline alone combination treatment with R7050(Fig. 3m, 3n, 3o).



**Figure 5.3.** Theophylline activates TNFR1 mediated apoptosis. After 6-hrs of theophylline treatment, qPCR as well as immunoblotting was performed for TNFR1 in MDA-MB-231 (a, b) and HEK293 (c, d) cells. Immunoblotting of (e) TRADD, (f) cRIP and (g) TNF $\alpha$  protein in MDA-MB-231 after 6-hrs theophylline treatment. Immunoblotting of cleaved caspase-8 (h), cleaved caspase-9 (i) and cleaved caspase-3 (j) apoptotic proteins was carried out after 6-hrs theophylline treatment.  $\beta$ -actin was used as an endogenous control. Graphs corresponding to immunoblots show average protein expression of three independent experiments normalized with respect to (w.r.t) endogenous control. (k) Mitochondrial membrane potential was measured using TMRE dye after 6-hrs theophylline treatment (l) Cytotoxicity was measured by MTT assay with and without R7050 after 24-hrs of theophylline treatment. (m, n, o) Level of cleaved caspase-8, -9 and

-3 proteins in control and theophylline treated cells were measured by immunoblotting in presence and absence of R7050.  $\beta$ -actin was used as an endogenous control. (C: control, T: theophylline; \* indicates  $p \le 0.05$ ).

### **5.2.4. ROS regulated TNFR1 expression contributes to the cytotoxic effects of theophylline on MDA-MB-231 breast cancer cells**

MDA-MB-231 breast cancer cells when treated with theophylline showed higher amount of ROS generation in comparison to control (fig. 4a). Cellular lipide peroxidation is usually followed by increased ROS generation, therefore we examined the level of lipid peroxidation via thiobarbituric acid reacting substances (TBARS) assay and we found the theophylline treatment increases amount of lipid peroxidation in comparison of the control treatment (fig. 4b). Next, ROS inhibitor NAC(N-acetyl-L-cysteine) was used to confirm the involvement of ROS in apoptosis induction after theophylline treatment. When cells were pre-treated with NAC, we observed a significant decrease in generation of ROS, followed by reduction in lipid peroxidation after theophylline treatment (Fig. 4c, 4d). Cells treated with combination of NAC and theophylline showed decreased cell death and increase in cell viability in comparison to the single treatment of theophylline (Fig. 4e, 4f). AO/EtBr staining also showed reduced number of apoptotic cells in NAC treated cells as compared to theophylline alone (Fig. 4g, 4h). Next, we examined the involvement of ROS in regulation of TNFR1 mediated apoptosis, interesting we found that NAC treatment reduced TNFR1 and cleaved caspase-3 protein level as compared to theophylline alone (Fig. 4i, 4j).



Figure 5.4. Theophylline induces ROS production inhibition of which results in decreased TNFR1 mediated apoptosis in MDA-MB-231 breast cancer cells (a) ROS was measured in presence of DCFDA reagent after 4-hrs theophylline treatment. (b) Oxidative damage to lipids was measured by TBARS assay after 6-hrs of theophylline treatment. (c) The intracellular ROS production was measured in control and theophylline treated cells by DCFDA assay in presence and absence of ROS inhibitor, NAC. (d) TBARS assay was used to measure the levels of lipid peroxidation in cells treated with/without theophylline in presence and absence of NAC. Percentage of cell death (e) and cell viability (f) in cells treated with/without theophylline in presence and absence of NAC was assessed using trypan blue and MTT assay, respectively. Early (g) and late (h) apoptosis was assayed using AO/ EtBr dye in control and theophylline treated cells in presence and absence of NAC. Levels of TNFR1 (i) and cleaved Caspase-3 (j) proteins in control and theophylline treated cells were measured by immunoblotting in presence and absence of NAC. The graphs show the average protein expression from three independent experiments normalized with respect to (w.r.t) endogenous control. (C: control; T: theophylline; \* indicates  $p \leq 0.05$ ).

#### 5.2.5. Binding of adenosine-to-adenosine receptor reduces ROS in MDA-MB-231 cell line

Theophylline is a well know antagonist of adenosine receptors, A1 and A2<sup>172–174</sup>. It has been shown that MDA-MB-231 cell line expresses A2 adenosine receptor<sup>175</sup>. Studies have shown theophylline and adenosine to compete for adenosine receptor when given in combination. It is also known that activation of adenosine receptor after binding with adenosine reduces  $ROS^{176,177}$ . In this context we studied the levels of ROS after treatment with theophylline and adenosine (200µM). The obtained intrinsic ROS level have reduced in combination treatment in comparison to theophylline alone (Fig. 5a). Interestingly, we even observed that when theophylline is present in combination with adenosine (200), cell death decreases and cell viability increases in comparison to theophylline individual treatment (Fig. 5b, 5c).



Fig. 5 Theophylline competes for adenosine receptor in MDA-MB-231 cells. (a) The intracellular ROS production was measured by DCFDA assay after treatment with theophylline in combination with adenosine. Percentage of cell death (a) and cell viability (b) in cells treated with theophylline in combination with adenosine was assessed using trypan blue and MTT assay, respectively. (C: control; T: theophylline; A1: Adenosine 200 $\mu$ M; \* indicates p≤0.05)

### **5.2.6.** TNFR1 causes upregulation of cathepsin activity in MDA-MB-231 breast cancer cells as a result of theophylline treatment

Cathepsin, a well-known lysosomal cysteine protease, has been reported to be part of TNF $\alpha$  mediated apoptosis pathway. We observed that when MDA-MB-231 cells were treated with theophylline for 6hrs, there is a significant increase in cathepsin level(p $\leq$ 0.05)

(Fig. 5a). Thereafter, treating the cells with TNFR1 blocker R7050 reduced the active cathepsin level after combination treatment with theophylline (Fig. 5b).



**Figure 5.6.** Theophylline treatment causes increased cathepsin activity which is reduced on inhibition of TNFR1 in MDA-MB-231 cells. (a) The activity of cathepsin was measured as the amount of fluorescent AMC dye after 6-hrs of theophylline treatment (b) The cathepsin activity showed reduction on treatment of the cells with TNFR1 inhibitor, R7050. All experiments were carried out in triplicate and repeated three times. Representative experiment is shown in the figure (C: control; Tr:rhTRAIL; T: theophylline; Tr+T: rhTRAIL+Theophylline; \* indicates  $p \le 0.05$ ).

### 5.2.7. rhTRAIL in combination with theophylline causes cytotoxicity in breast cancer cell line and reduced cell migration

As we have already established that theophylline has cytotoxic effect on MDA-MB-231 TNBC cell line, now we are trying to study the effect of rhTRAIL in combination with theophylline. Here we observed via trypan blue and MTT assay that combination of 150ng/ml of rhTRAIL with 10mM of theophylline induces significant cell death in comparison to rhTRAIL and theophylline alone after 24hrs of treatment( $p\leq0.05$ ) (Fig. 6a, 6b). Late apoptosis caused by combination treatment was observed by EtBr-stained red nucleus after 6hrs of treatment, experiment showed similar results as the earlier assay, in combination the percentage of late apoptotic cells were greater that those of rhTRAIL and theophylline treated( $p\leq0.05$ ) (Fig. 6c, 6d). Interestingly we also observed in scratch assay that rhTRAIL in combination reduces cell migration after 24hrs of treatment significantly higher that rhTRAIL or theophylline alone ( $p\leq0.05$ ) (Fig. 6e, 6f).





### 5.2.8. Theophylline in combination with rhTRAIL induced both extrinsic and intrinsic pathways of apoptosis in MDA-MB-231 breast cancer cells

Next, we observed that theophylline in combination with rhTRAIL significantly increases the primary effector caspase i.e. cleaved caspase-8 protein level after 6 h of treatment in comparison to theophylline or rhTRAIL alone (Fig. 7a). TRAIL is well known for effecting both the branches, therefore we studied the levels of intrinsic apoptotic gene cleaved caspase-9 and the final effector molecule of caspase cascade cleaved caspase-3. Both the caspases were significantly upregulated in theophylline and rhTRAIL alone as well as in combination compared to control cells. There was even significant upregulation in the level of the cleaved caspase-9 and caspase-9 and caspase-3 in combination treatment than those of theophylline and rhTRAIL treated sample (Fig. 7b, 7c).



**Figure 5.8.** Combination treatment triggers intrinsic apoptotic pathway. .(a, b,c) Level of cleaved caspase-8, -9 and -3 proteins in 6hrs treated cells were measured by immunoblot.  $\beta$ -actin was used as an endogenous control. (C: control; Tr:rhTRAIL; T: theophylline; Tr+T: rhTRAIL+Theophylline; \* indicates  $p \le 0.05$ ).

5.2.9. Combination treatment induced high ROS production along with reduced mitochondrial membrane potential and increased LPO in comparison to theophylline or rhTRAIL alone

To better understand the mechanism of cytotoxicity mediated via theophylline in combination with rhTRAIL, we checked the level of ROS production in MDA-MB-231 cells treated with theophylline and/or rhTRAIL. On combination treatment ROS generation was found to be significantly upregulated in cells, in comparison to rhTRAIL and theophylline separately (Fig. 8a). We also observed mitochondrial membrane potential

to be lower and lipid peroxidation level to be higher in combination treatment compared to single treatment of rhTRAIL and theophylline (Fig. 8b, 8c).



**Figure 5.9.** Oxidative stress was measured after the treatment of MDAMB-231 cells with theophylline and/or rhTRAIL (d) ROS was measured in presence of DCFDA reagent after 4-hrs theophylline treatment and combination treatment. (e) Mitochondrial membrane potential was measured using TMRE dye after 6-hrs of rhTRAIL, theophylline and combination treatment (C: control; Tr:rhTRAIL; T: theophylline; Tr+T: rhTRAIL+Theophylline; \* indicates  $p \le 0.05$ ).

### 5.2.10. TRAIL in combination with theophylline increase expression of Death receptor 5

In order to explore the molecular mechanism activated by combination treatment we studied death receptors DR5 expression level as activation of apoptosis cascade starts with TRAIL binding to its receptors. DR5 which is a known death receptor of TRAIL is surprisingly upregulated after 6hrs of combination treatment ( $p \le 0.05$ ) (Fig. 9a).



**Figure 5.10.** Upregulation of death receptors by rhTRAIL in combination with theophylline. (a) Expression levels of DR5 protein in 6hrs treated cells were measured by immunoblot.  $\beta$ -actin was used as an endogenous control. (C: control; Tr:rhTRAIL; T: theophylline; Tr+T: rhTRAIL+Theophylline; \* indicates p≤0.05).

#### 5.2.11. Discussion

Apoptosis is one of the natural machineries, whose maintenance decides the well-being of a multi-cellular organism. During the evolutionary process the morphological and biochemical characteristics of apoptosis has remain conserved. However, during carcinogenesis this mechanism gets disrupted thereby resulting in tumor formation. Over the past few decades, theophylline, a methylxanthine drug has been used for treating respiratory diseases such as asthma because of its immunosuppressive properties<sup>167</sup>. When theophylline was used in combination therapy, it showed promising results for AML patients in clinical trials<sup>170</sup>. Lately it has been shown to have a potential in treating breast cancer. Previously it has been shown that theophylline induces increase of the intracellular cAMP concentration, which led to a decrease in mitosis in breast cancer cell lines<sup>166</sup>. Chang et.al, 2017 has shown cytotoxic effect of theophylline on MDA-MB-231 breast cell line but MCF10A normal breast cancer cells were not at all effected by theophylline treatment<sup>168</sup>.

In the present study, we demonstrate capacity of theophylline to induce cytotoxicity via TNFR1 mediated apoptosis in triple negative MDA-MB-231 breast cancer cells. 10mM of theophylline dose is chosen via a dose dependent MTT assay, which results in 50% of cellular toxicity in MDA-MB-231 breast cancer cell line and also in tumorigenic embryogenic kidney HEK293 cell line but not in 'normal' immortalized mouse embryonic fibroblast cells, NIH3T3 (Fig. 1a. 1b, 1c). Theophylline 10mM dose has shown a significant increase in MDA-MB-231 cell death (Fig. 2a). DNA damage is often associated with cell death; therefore, we have performed both types of comet assays (alkaline and neutral) to identify if the apoptotic cells show any form of DNA damage. Theophylline exposure causes increased DNA damage as well as double strand DNA breaks in comparison to control (Fig. 2b, 2c). When treated cells were stained with acridine orange (AO) and ethidium bromide (EtBr) some typical morphological changes occurred in those cells. Microscopic study has clearly signs of apoptotic bodies, membrane blebbing and chromatin condensation which are the markers of early apoptosis (Fig. 2d). The level of

late apoptotic cells were also high after treatment with theophylline in comparison to the control cells (Fig. 2e, 2f).

Next, we tried to figure out the molecular mechanism underling the apoptosis induced by theophylline treatment. TNFR1 receptor was upregulated in both transcript and protein levels by theophylline in MDA-MB-231 breast cancer cells as well as tumorigenic HEK293 cells that contain adenovirus 5 DNA (Fig. 3a, 3b, 3c, 3d). One of the reported pathways of ROS production is the activation of TNFR1, TRADD and RIP complex<sup>178–180</sup>. TNFR1-associated death domain protein (TRADD) is the first adaptor molecule of activated TNFR1 signaling pathway<sup>181,182</sup>. It is an essential molecule for recruitment of TRAF2 and RIP. Study has shown TRADD, TRAF2 and RIP recruits NOX1 of the NADPH oxidases family followed by production of ROS<sup>183</sup>. Interestingly, in our study we observed that TRADD, cleaved RIP and TNF $\alpha$  of the TNFR1 complex are also upregulated at the protein level after theophylline treatment (Fig. 3e, 3f, 3g)

Caspases are the executioners of apoptosis; theophylline upregulates the protein level of cleaved caspase-8 which acts as the initiator molecule for effector caspase 3(Fig. 3h, 3j). We even observe elevated levels of cleaved caspase 3 after theophylline treatment in MDA-MB-231 cells. Reduction in mitochondrial membrane potential was also observed after theophylline treatment, including increase in amount of cleaved caspase 9 (Fig. 3i, 3k). Theophylline treatment showed increase in lipid peroxidation level in comparison to control, indicating the presence of oxidative stress. When cells were pre-treated with TNFR1 inhibitor R7050, they show less cell death in compassion to theophylline individual treatment. (Fig. 3l) Even the expression level of cleaved caspase-8, 9 and 3 reduced in present of R7050 inhibitor, when compared to theophylline treatment (Fig. 3m, 3n, 3o). Thereby, we can conclude that both extrinsic as well as intrinsic apoptotic pathway were activated by theophylline via TNFR1 in MDA-MB-231 breast cancer cells.

Further, we try to evaluate the impact of theophylline on ROS production in breast cancer cells as we have already seen increased amount of oxidative damage. The chosen dose of theophylline has shown to increase the ROS and LPO levels in MDA-MB-231 cells after 6hrs of treatment (Fig. 4a, 4b). To establish that ROS induces cell death in theophylline treated breast cancer cells, cells were cotreated with ROS inhibitor NAC. As NAC significantly reduced the level of ROS and LPO in theophylline treated cells, as a result the cell death percentage significantly reduced and simultaneously cell survival percentage

has increased (Fig. 4c, 4d, 4e, 4f). ROS mediated TNFR1 increase in mouse neuronal cells was shown by Ma et.al. NAC cotreatment with theophylline even inhibits the morphological changes exhibited by the only theophylline treated cells, thereby reducing amount of early and late apoptotic cells (Fig. 4g, 4h). ROS activates the cell death pathway via TNFR1 receptor, as after NAC cotreatment both TNFR1 and cleaved caspase 3 levels reduces significantly in comparison to theophylline treatment alone. (Fig. 4i, 4j). We also observed a feedback mechanism of TNFR1 with ROS, in the context of theophylline, as after treatment with TNFR1 inhibitor R7050 the amount of intracellular ROS production was significantly hampered (Fig. 11a). Thus, our results shows theophylline causes upregulation in ROS production which in turns results in the activation of TNFR1 mediated apoptosis via caspase-3.

Studies have shown theophylline as an antagonist for adenosine receptor, it is known to compete with adenosine for adenosine receptors, A1and A2<sup>172–174</sup>. When adenosine binds with its respective adenosine receptors, it reduces intracellular ROS levels<sup>176–178</sup>. MDA-MB-231 TNBC cell line is known for expressing A2 adenosine receptor on its surface<sup>175</sup>. Thereby, we were interested to study regulation of ROS in MDA MB 231 cells in presence of theophylline and/or adenosine. We observed that the intracellular ROS level had significantly reduced when theophylline was given in combination with adenosine in comparison to the theophylline alone (Fig. 5a). We even observed that when theophylline is added in combination with adenosine given in increased doses, cell death decreases and cell viability increases (Fig. 5b, 5c). Therefore, we can conclude that theophylline increases ROS which is reduced in presence of adenosine.



**Figure 5.11.** TNFR1 inhibitor R7050 causes reduction in ROS level after theophylline treatment in MDA-MB-231 cells. (a) The intracellular ROS production was measured in control and theophylline treated cells by DCFDA assay in presence and absence of TNFR1 inhibitor, R7050. (C: control; Tr:rhTRAIL; T: theophylline; Tr+T: rhTRAIL+Theophylline; \* indicates  $p \le 0.05$ ).

Another novel cell death pathway that is being activated by theophylline in breast cancer cells is cathepsin mediated apoptotic pathway. Cathepsin, which is a lysosomal protease under certain physiological circumstances help in activation of apoptosis. It has been already shown that activation of TNFR1 induces lysosomal rupture through production of sphingosine, which permeabilizes the lysosome membrane and localizes the lysosomal enzymes to cytosol. We have already seen that theophylline treatment increases TNFR1 protein expression in breast cancer cells, which in turns helps in lysosomal rupture and release of cathepsin in cytosol of the theophylline treated cells (Fig. 6a, 6b). It has been also reported that higher level of ROS facilitated lysosomal rupture in certain cancer cells. However, in our study we did not observe any effect on cathepsin activity after ROS inhibition (Fig. 12a).



**Figure 5.12.** ROS did not show any effect of cathepsin activity after theophylline treatment in MDA-MB-231 cells. (a) The activity of cathepsin was measured as the amount of fluorescent AMC dye after 6-hrs of theophylline treatment (C: control; Tr:rhTRAIL; T: theophylline; Tr+T: rhTRAIL+Theophylline; \* indicates  $p \le 0.05$ ).

In conclusion, we have treated MDA-MB-231 breast cancer cells with theophylline to demonstrate that increased ROS production is responsible for activation of both the extrinsic and intrinsic arms of apoptosis via TNFR1 death receptor (Fig. 13). We can even hypothesize that TNFR1 forms a complex with TRADD, RIP and TRAF2, which in turn recruits the NADPH family members thereby resulting in formation of ROS after

theophylline treatment, thereby creating a feedback loop between TNFR1 and ROS generation.

We also hypothesis that activation of caspase 8 via TNFR1 receptors mediates increase in cathepsin activity, which in turn might enhances apoptosis. Therefore, or study indicates theophylline could be possibly used as a potential therapeutic durn in context of breast cancer.



**Figure 5.13**. Schematic diagram showing the signalling pathway activated by theophylline in MDA-MB-231 breast cancer cells. Theophylline on entering the cell results in increased ROS production. High ROS causes increased TNFR1 expression, which in the presence of TNFA ligand, activates caspase-mediated apoptosis. ROS is also generated as a product of TNFA-TNFR1 signaling pathway. Furthermore, TNFA on binding to TNFR1 causes cleavage and activation of caspase-8 thereby initiating extrinsic apoptotic pathway. Next, cleaved caspase-8 is responsible for cleavage and activation of caspase-3. Active caspase-8 is also responsible for increasing active cathepsin which in turn promotes apoptosis. Likewise, theophylline causes the initiation of intrinsic apoptotic pathway by activating caspase-9 which in turn is also capable of activating caspase-3. (c. Casp8: cleaved caspase 8; c. Casp9: cleaved caspase 9; c. Casp3: cleaved caspase 3).

In search for alternative combination drug therapy, theophylline's toxic nature to the cancerous cells has opened up a new path of possibilities. TRAIL is regarded as a potential therapeutic agent because of its promising anti-cancer properties. TRAIL sensitivity ranges in various cancer types and the most malignant tumors being resistant to TRAIL treatment gives more reason for finding new molecules to sensitize these cells towards TRAIL induced apoptosis. In the present study we observed that theophylline in combination with rhTRAIL is responsible increased cell death and decrease in cell viability in treated MDA-Mb-231 breast cancer cells in comparison to theophylline or rhTRAIL individual treatments (Fig. 7a, 7b). The amount of late apoptotic cells have also increased in combination treatment in comparison to theophylline and rhTRAIL alone (Fig. 7c, 7d). Interestingly we also observed decreased cell migration in combination treatment when compared to the individual tretmnets (Fig. 7e, 7f). The extrinsic apoptotic pathway was activated via upregulation of cleaved caspase-8 in rhTRAIL, theophylline and combination treated cells (Fig. 8a). To confirm the activation of intrinsic pathway, we preformed western blot assay for caspase-9 and the final molecule of apoptotic pathway caspase-3. Cleaved form of both the caspases were significantly upregulated in cells treated with combination of theophylline and rhTRAIL in comparison to rhTRAIL and theophylline alone (Fig. 8b, 8c).

ROS generation disrupts lipid by- layer and the mitochondrial membrane potential, causing activation of intrinsic apoptotic pathway. We investigated the ROS production level in cells treated with combination of rhTRAIL and theophylline where we observed that the amount of ROS is significantly higher in combination with respect to rhTRAIL and theophylline alone (Fig. 9a). In continuation of the pathway, we also found LPO level to be higher and mitochondrial membrane potential level was lower in cells treated with the combination dose (Fig. 9b, 9c). There are reports stating upregulation of DR5 sensitizes tumor cells towards TRAIL mediated apoptosis. Interestingly, we observed that DR5 protein expression level was upregulated in combination treated cells in comparison to rhTRAIL and theophylline alone treatment (Fig. 10a). Repots have already claimed that ROS upregulates DR5 expression in multiple cancer types and DR5 is capable of activating both extrinsic and intrinsic apoptotic pathway. Taken together, we hypothesis that combination elevates ROS up to a threshold that results in upregulation of DR5 expression.

The increase in abundance of death receptor after combination treatment, enhances the chance of TRAIL binding of its receptor thereby activating more of apoptosis cascade (Fig. 14).





In conclusion we can say that theophylline in combination with rhTRAIL causes significant increase in cytotoxicity via upregulation of DR5 receptor causing higher amount of apoptosis in comparison to individual rhTRAIL or theophylline treatment. This is the first study reporting that TRAIL in combination with theophylline can be a novel strategy for treating triple negative breast cancer patients.