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# Phenothiazine derivatives and their impact on the necroptosis and necrosis processes. A review

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ABSTRACT

The current review focuses on the effect of phenothiazine derivatives, tested *in vitro*, on necrosis and necroptosis, the latter constitutes one of the kinds of programmed cell death. Necroptosis is a necrotic and inflammatory type of programmed cell death. Phenothiazines are D1 and D2-like family receptor antagonists, which are used in the treatment of schizophrenia. Necroptosis begins from TNF-α, whose synthesis is stimulated by dopamine receptors, thus it can be concluded that phenothiazine derivatives may modulate necroptosis. We identified 19 papers reporting *in vitro* assays of necroptosis and necrosis in which phenothiazine derivatives, and both normal and cancer cell lines were used. Chlorpromazine, fluphenazine, levomepromazine, perphenazine, promethazine, thioridazine, trifluoperazine, and novel derivatives can modulate necroptosis and necrosis. The type of a drug, concentration and a cell line have an impact on the ultimate effect. Unfortunately, the authors confirmed both processes on the basis of TNF-α and ATP levels as well as the final steps of necrosis/necroptosis related to membrane permeability (PI staining, LDH release, and HMGB1 amount), which makes it impossible to understand the complete mechanism of phenothiazines impact on necroptosis and necrosis. Studies analyzing the effect of phenothiazines on RIPK1, RIPK3, or MLKL has not been performed yet. Only the analysis of the expression of those proteins as well as necrosis and necroptosis inhibitors can help us to comprehend how phenothiazine derivatives act, and how to improve their therapeutic potential.

## 1. Introduction

Necroptosis can be defined as a form of programmed cell death, which is a caspase-independent and pro-inflammatory process (Tan et al., 2018). Necroptosis may be effectively used in cancer therapy since it does not only bypass apoptosis resistance and maintains antitumor immunity but, in addition, cancer progression, metastasis, and immunosurveillance are related to it (Wu et al., 2020). In contrast, necrosis is defined as an uncontrolled process, thus it is very hard to find tiny molecules interfering with it (Wu et al., 2020), and it is characterized by stochastic membrane lysis (Atkin-Smith, 2021). Necroptosis and necrosis share one feature, which means their membrane integrity can be lost, however, the permeabilization of a cellular membrane is strictly regulated in necroptosis (Tan et al., 2018; Vanden Berghe et al., 2010).

In the early stage of apoptosis, mitochondrial outer membrane permeabilization leads to cytochrome *c* release into cytosol (pro-apoptotic factor), but swelling of the matrix (model of mitochondrial permeability transition pore activation) is not observed in all apoptosis scenarios. Moreover, mitochondrial outer membrane permeabilization sometimes is observed in the late stage of apoptosis after the release of cytochrome *c* and caspase activation. Thus, it suggests that mitochondrial outer membrane permeabilization does not initiate apoptosis, but it is mainly involved in necrosis. Interestingly, depending on cell type and death stimulus, mitochondrial outer membrane permeabilization may cause swelling and cristae remodeling leading to cytochrome *c* and other proapoptotic factors release from mitochondria. It is possible since mitochondrial apoptosis-induced channel and mitochondrial outer membrane permeabilization may act singly or in combination (Kinnally

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et al., 2011). The morphological features of necrosis are "translucent cytoplasm, swelling sub-cellular membrane organelles, increased cell volume, early loss of membrane integrity, and spillage of intracellular contents into extracellular space". In contrast, apoptosis is characterized by morphological features such as "cytoplasmic shrinkage, chromatin condensation, DNA fragmentation, cell membrane blebbing, as well as apoptotic body formation and shedding" (Chaouhan et al., 2022).

First-generation antipsychotics, for example phenothiazine derivatives, are the representatives of heterocyclic compounds. In phenothiazines, there is the dialkylaminoalkyl substituent at position 10, and a small substituent at position 2 of the thiazine nitrogen atom. This group of drugs is used in the clinical treatment of psychotic disorders (Morak-Młodawska et al., 2021). Phenothiazine derivatives have numerous activities, and perform many biological functions (Morak-Młodawska et al., 2021; Otręba et al., 2020), of which their anticancer effect is worth mentioning (Morak-Młodawska et al., 2021; Otręba and Kośmider, 2021).

Phenothiazines are capable of modulating necroptosis since they are antagonists of D1 and D2-like family receptors (Bandala et al., 2023; Froimowitz and Cody, 1993; Smith et al., 2012 Varga et al., 2017). The activation of D2 and D3 receptors (Rangel-Barajas et al., 2015) is necessary for the synthesis of TNF-α, which is a TNF cytokine responsible for necroptosis activation (Wang et al., 2022; Wu et al., 2020). Moreover, dopamine affects D1, D5, and D3 receptors, and consequently increases the production of TNF-α (Rangel-Barajas et al., 2015). Wu et al. (2022) suggested that a decrease of necroptosis in acute pancreatitis cells treated with quinpirole, *i.e.* D2 receptor agonist, may be caused by inhibiting cathepsin B, which stimulates necroptosis by degradation of mitochondrial transcription factor A. Thus, the activation of D2 inhibits cathepsin B and reduces necroptosis (Wu et al., 2022). Interestingly, phenothiazine derivatives are also able to induce mitochondrial permeability transition facilitating the cytochrome *c* release (Cruz et al., 2010, de Faria et al., 2015), and changing the properties of plasma membrane (Heitmann et al., 2021, de Faria et al., 2015). One of the mechanism suggests that the specific thiol groups and chemically generated phenothiazine cation radicals can interact in the presence of calcium ions (Cruz et al., 2010). Moreover, de Faria et al. observed that (i) the side chain of phenothiazines is key for cytotoxic potential, (ii) phenothiazines promote plasma membrane permeabilization, and (iii) there is a close correlation between the cytotoxicity and mitochondrial transmembrane potential. When cytotoxicity is concerned, the following order from the most to the least cytotoxic substance can be suggested: piperidinic (thioridazine), piperazinic (fluphenazine and trifluoperazine), and aliphatic derivatives (chlorpromazine and trifluopromazine). For plasma membrane permeabilization efficiency, the order can be as follows, from the most to the least efficient substance: trifluoperazine, thioridazine, and trifluopromazine. In the case of dissipation of the mitochondrial transmembrane potential, thioridazine was most effective, fluphenazine was less effective and trifluopromazine was the least effective substance (de Faira et al., 2015). Furthermore, Heitmann et al. (2021) reported that phenothiazine derivatives act as pan-members of the Annexin inhibitors leading to disturbance of the repair of the plasma membrane. It is important because, as we mentioned above, loss of the integrity of the plasma membrane is related to necrosis and necroptosis.

This current review is focused on the effect of phenothiazine derivatives on necroptosis and necrosis, including their molecular mechanisms and *in vitro* assays.

## 2. Materials and methods

In June 2022, PubMed and Google Scholar were searched for English-language papers containing the phrases "(cancer) OR (tumor) AND (cell line) AND (necrosis) AND (phenothiazine) OR (chlorpromazine) OR (fluphenazine) OR (perphenazine) OR (prochlorperazine) OR (promethazine) OR (thioridazine) OR (trifluoperazine)" resulting in 453 records from Google Scholar and 272 records from PubMed as well as "(cancer) OR (tumor) AND (cell line) AND (necroptosis) AND (phenothiazine) OR (chlorpromazine) OR (fluphenazine) OR (perphenazine) OR (prochlorperazine) OR (promethazine) OR (thioridazine) OR (trifluoperazine)" resulting in 43 records from Google Scholar and 25 records from PubMed. Totally, 40 papers published between 2000 and 2021 were considered appropriate for a further analysis, and we based our choice on the titles and abstracts. Consequently, 22 papers were rejected. We employed the same inclusion and exclusion criteria as in the previous review (Otręba et al., 2022), however, this time they referred to necrosis and necroptosis. About the type of the cell, we accepted all *in vitro* studies using human or animal cancer and normal cell lines (as a control, if only they were used). The remaining articles were divided into 6 groups based on cell type. There was 1 paper each about breast, circulatory system, and liver; 2 papers each about pancreas, sex organs, and skin; 3 papers about the immune system; 4 papers about brain as well as 2 about the *in vivo* studies (Fig. 1). Finally, we selected 18 original articles published between 2000 and 2021 for our analysis.

## 3. Phenothiazine derivatives' effect on necrosis

### 3.1. In vitro studies

#### 3.1.1. Brain

Uzdensky et al. (2007) measured the impact of fluphenazine (1 μM) as a calmodulin inhibitor on necrosis in isolated crayfish neurons and satellite glial cells. The authors observed that fluphenazine reduced necrosis of neurons and glial cells. The fact that calmodulin was inhibited by fluphenazine did not significantly affect sensory neuron activity (in the dark and after photodynamic therapy - PDT). In the absence of light, after 360 ± 50 min in the presence of fluphenazine, the neuronal activity was terminated, when compared with 440 ± 40 min without the drug. Moreover, the authors did not record any crucial effect of fluphenazine on voluntary necrosis of stretch receptor neurons and glial cells in the dark, while necrosis of neurons (from 77% ± 12% without fluphenazine to 29% ± 11% when it was used) and glial cells (from 74% ± 4–41% ± 9%, respectively) was significantly reduced after PDT-therapy. The obtained % of necrotic neurons were about 20%, 15%, 80%, and 30%, respectively for the control, fluphenazine (1 μM), PDT, as well as fluphenazine (1 μM) and PDT. In the case of glioma cells, the obtained % of necrotic cells were approximately 30%, 20%, 75%, and 40%, respectively for the control, fluphenazine (1 μM), PDT, as well as fluphenazine (1 μM) and PDT (Uzdensky et al., 2007). Therefore, it can be assumed that calmodulin is involved in the necrosis of stretch receptor neurons and glial cells, and that fluphenazine decreases in necrosis of neurons and glial cells (Uzdensky et al., 2007).

The effect of perphenazine and trifluoperazine on necrosis of human neuroblastoma SH-SY5Y cells was studied by Hong et al. (2012). The researchers noted a significant reduction in ATP levels after 8 and 16 h of incubation of neuroblastoma cells with perphenazine (25 μM) and trifluoperazine (25 μM). There was observed 80% and 40% as well as 90% and 60% reduction of ATP level, respectively for 8 and 16 h-incubation of perphenazine and trifluoperazine, which confirms necrosis because the reduction of ATP level in the cells and oxidative stress may facilitate necrotic cell death. The authors found that "perphenazine and trifluoperazine can induce apoptotic cell death *via* mitochondrial damage, but inhibition of caspases possibly shifted the cell death mode to necrosis" (Hong et al., 2012).

The effect of thioridazine on necrosis of human glioblastoma U-87 MG and GBM8401 cells was studied by Chu et al. (2019). The researchers used PI flow cytometry and annexin V staining method. The obtained results showed that incubation of U-87 MG cells with thioridazine 0, 5, 10, and 15 μM lead to 12.39%, 6.39%, 7.13%, and 13.92% of late apoptotic or necrotic cells, respectively. In the case of GBM8401, the authors observed 6.74%, 4.96%, 13.09%, and 29.95% of late apoptotic or necrotic cells, respectively (Chu et al., 2019). Based on the results, it

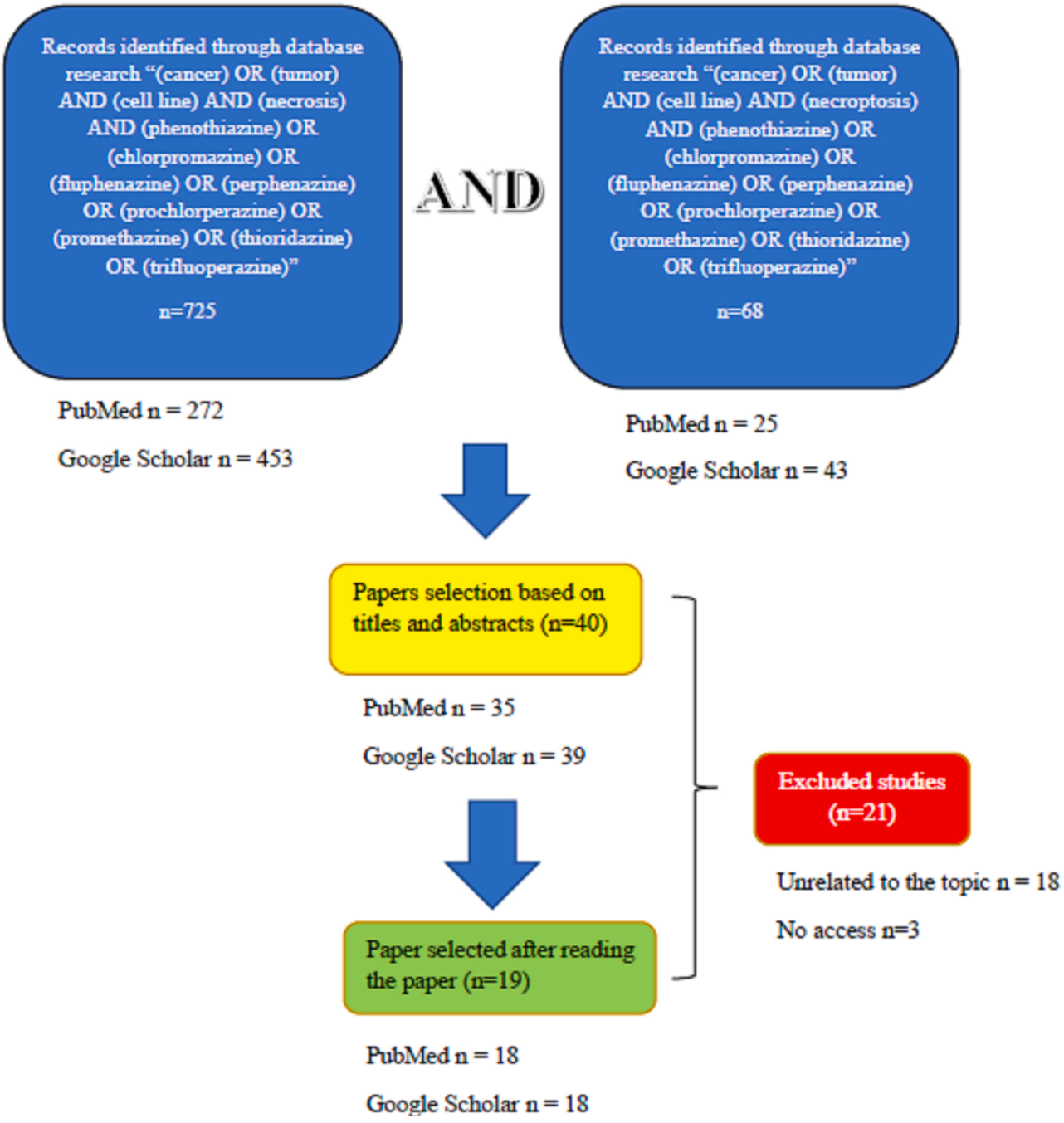


**Fig. 1.** The process of selection of the papers.

can be suggested that treatment of glioblastoma cells with thioridazine may cause necrosis or late apoptosis.

The impact of a novel phenothiazine derivative (DS00329) on necrosis of human glioblastoma U-87 MG and U-251 cells was studied by Omoruyi et al. (2020). The authors used a PI flow cytometry assay and annexin V staining. In U-87 MG cells treated by DS00329 for 24 and 48 h, the number of necrotic cells was almost the same as in the control. The authors observed slightly more necrotic cells in U-251 cells treated with DS00329 for 24 and 48 h (Omoruyi et al., 2020) when the results were compared to the control.

### 3.1.2. *Immune system*

Jurkat cells and rat peripheral blood mononuclear cells (PMBC) after chlorpromazine (50, 300, and 500 ng/ml) treatment and UVA irradiation (200 kJ/m2) were used to measure necrosis by Wolnicka-Głubisz et al. (2002). Flow cytometric and fluorescence microscopy analyses were performed. The authors observed a similar amount of apoptotic and necrotic cells after chlorpromazine treatment (300 and 500 ng/ml), while in chlorpromazine treated (300 ng/ml) and UVA irradiated cells there was observed a significant increase in the number of apoptotic and necrotic cells. Flow cytometry of Jurkat cells treated with chlorpromazine 50, 300, and 500 ng/ml) and irradiated with UVA showed an increase from about 25–35% of propidium iodide (PI) positive cells. The fluorescence microscope analysis of Jurkat cells stained with acridine orange and propidium iodide showed the presence of orange-stained cells with a normal appearing nucleus (treated as necrotic cells) (Wolnicka-Głubisz et al., 2002), which suggests that chlorpromazine may stimulate necrosis in human and rat lymphocytes.

The effect of 10 novel fluphenazine derivatives on necrosis of human lymphocytes genotoxically damaged by incubation with B[*a*]P (7.5 μM, 48 h) was analyzed by Zyta et al. (2014). Annexin V-FITC/propidium iodide staining kit was used, and then the cells were analyzed under a fluorescence microscope. Necrotic cells emitted red fluorescence, the apoptotic emitted green fluorescence, while viable cells were not stained. Compounds 3, 9, and 10 had the weakest influence on the frequency of necrotic lymphocytes in cultures. The authors also observed that the apoptosis/necrosis index was about 10–15 times lower than for fluphenazine and compounds 5, 10, and 11, therefore it can be concluded that in those compounds necrosis was stronger. Moreover, $TD_{50}$ (pronecrotic effect, cytotoxicity) was also calculated. The obtained values of $TD_{50}$ were 46.88, 29.41, 14.89, 25.87, 46.37, 32.02, 11.02, 13.91, 102.85, and 16.97 μM, respectively for compounds 3–12 and fluphenazine, consequently compounds 3, 4, 6, 7, 8, and 11 are pronecrotic (Zyta et al., 2014).

Tsuji et al. (2021) studied the effect of perphenazine (20 μM) on necrosis of the human Sezary syndrome cell line (HUT78). The authors used PI flow cytometry with annexin V staining. Approximately 30% late apoptotic/necrotic cells after perphenazine treatment in comparison to the control (Tsuji et al., 2021) were observed.

### 3.1.3. Pancreas

Huang et al. (2019) showed that trifluoperazine (10 and 20 μM) caused apoptotic cell death of pancreatic cancer MiaPaCa-2 cells, and necroptosis resulting from a combined stress of endoplasmic reticulum and mitochondrial. The authors used flow cytometry after staining cells with propidium iodide (PI) and annexin V treated with TFP. They observed a dose-dependent increase of PI-positive cells from 3.62% ± 0.15% in non-treated cells to 22.08% ± 2.60% or 46.63% ± 1.33%, respectively for 10 and 20 μM of trifluoperazine. To confirm pro-necrotic activity, lactate dehydrogenase (LDH) release was measured. The obtained results showed a strongly increase in trifluoperazine-treated cells. It is worth observing that using Z-VAD-FMK or the necroptosis inhibitor (Necrostatin-1) reduced the percentage of PI stained cells from 22.08 ± 2.60% in trifluoperazine (10 μM) treated cells to 13.62 ± 0.72% when the cells were co-treated with Necrostatin-1, or 15.84 ± 0.81% when they were co-treated with Z-VAD. The authors also noticed that trifluoperazine reduced ATP content in a dose-dependent manner. In control samples, ATP produced by the OXPHOS metabolism was 92.17 ± 1.95 pmoles/min/1000 cells, while it was 54.23 ± 3.38 pmoles/min/1000 cells in trifluoperazine treated cells. Moreover, in control cells, ATP produced by glycolysis was 15.01 ± 0.188 pmoles/min/1000 cells, while in trifluoperazine treated cells it was 26.51 ± 1.86 pmoles/min/1000 cells (an increase by 32.83% of the total ATP produced upon treatment). The observed lactate release corresponds to an increased glycolytic metabolism (Huang et al., 2019), which suggests that trifluoperazine induces necrosis of MiaPaCa-2 cells.

The effect of trifluoperazine (ZZW-111) and six trifluoperazine derivatives (ZZW-112, ZZW-115, ZZW-116, ZZW-119, ZZW-120, and ZZW-124) on necrosis of pancreatic cancer cells: MiaPaCa-2 (a traditional cell line), 02.063 and LIPC (basal subtype), Foie8b (derived from a liver metastasis), and HN14 (classical subtype) was analyzed by Santofimia-Castaño et al. (2019). The authors measured LDH release and PI flow cytometry and annexin V staining. A substantial concentration-dependent rise in LDH release was recorded in ZZW-115 treated (0, 3, and 5 μM) cells in comparison to the controls. The authors observed also an increase in necrotic cells in ZZW-115 treated (0.5–5 μM) cells. The number of necrotic cells was 3.9%, 5.6%, 7.4%, 6.4%, and 9.6% for control samples, and 53.7%, 61.0%, 23.3%, 23.0%, and 44.4% for ZZW-115 treated cells, respectively for MiaPaCa-2, LIPC, Foie8b, 02–063, and HN 14 cells. It is worth noting that pretreatment of MiaPaCa-2 cells with the pan-caspase inhibitor Z-VADFMK and/or the necrosis inhibitor Nec-1 (prevent LDH release) induced a reduction in LDH release in a 5-μM ZZW-115 treated cells to 70%, 52%, and 46%, respectively for Z-VAD-FMK, Nec-1 and the combination of both inhibitors (Santofimia-Castaño et al., 2019). Such results indicate that independent mechanisms are involved when necroptosis and apoptosis are caused by ZZW-115.

### 3.1.4. Sex organs

Mao et al. (2015) studied the effect of thioridazine (20 μM) on the necrosis of human cervical squamous carcinoma SiHa cells. The researchers measured high-mobility group box 1 protein (HMGB1) to detect necrosis. HMGB1 is a biomarker of cell necrosis. The obtained values of HMGB-1 were 12.0 ± 2.0 and 35.1 ± 1.8 ng/ml, respectively for control and thioridazine samples, which suggests that necrosis caused the inhibition of SiHa cell growth (Mao et al., 2015).

Menilli et al. (2019) analyzed the impact of trifluoperazine (0, 2, 5, and 10 μM) with and without UVA irradiation (1.2 $J/cm^2$) on necrosis of human cervical adenocarcinoma cells (HeLa cells). The researchers used PI flow cytometry and annexin V staining. The number of necrotic cells was from 1.3% to 1.0% for trifluoperazine (0,2, and 5 μM) as well as 5.0–8.4% for trifluoperazine (0,5, and 10 μM) (Menilli et al., 2019), which indicates that necrotic cells constitute only a small fraction of trifluoperazine-treated HeLa cells.

### 3.1.5. Skin

Necrosis of immortalized human keratinocyte cell line (HaCaT cells) after treatment with different chlorpromazine concentrations was researched by Kurita et al. (2007). Chlorpromazine in the concentration range from $10^{-7}$ to $10^{-4}$ M with and without UVA irradiation (4 $kJ/cm^2$) was analyzed. Chlorpromazine $10^{-4}$ M with UVA irradiation significantly induced necrosis (about 80% cells) rather than apoptosis, while chlorpromazine $10^{-4}$ M without UVA increased the number of necrotic cells to about 40% (Kurita et al., 2007).

Kuzu et al. (2017) analyzed how fluphenazine and perphenazine (10 μM) affected necrosis of human melanoma UACC 903 cells. Necrosis was determined by annexin V PI staining. The authors observed a rise in necrotic cell amount after perphenazine treatment. The obtained results were 28.6% for Annexin V- PI+ (late necrosis) and 27.3% for Annexin V+ PI+ (early necrosis) for perphenazine-treated cells. In the control, the values were 0.6% and 0.4%, respectively. It can be highlighted that neither IM-54 nor Necrostatin-5 (necrosis inhibitors) protected cells from ASM inhibitors or leelamine-mediated cell death (Kuzu et al., 2017).

### 3.1.6. Other studies

During the analysis of the collected references, we found a few papers related to single assays using breast cancer cells (Seo and Efferth, 2016), human whole blood culture (Zimecki et al., 2009), myeloma cells (Li et al., 2020), hepatocytes and Kupffer cells (Hoebe et al., 2000). The impact of levomepromazine (0, 12.5, and 25 μM) on necrosis of human breast cancer MCF-7 was analyzed by Seo and Efferth (2016). The authors used Annexin V- PI staining. No cells were observed in a field of Annexin V- PI+ (late necrosis) and Annexin V+ PI + (early necrosis). Thus, the authors showed that levomepromazine was not cytotoxic since it caused neither necrosis nor apoptosis (Seo and Efferth, 2016).

The impact of chlorpromazine and 10 H-2,7-diazaphenothiazine on TNF-α in humans (human whole blood culture) was studied by Zimecki et al. (2009). 10 H-2,7-diazaphenothiazine was proven to inhibit the lipopolysaccharide-induced production of a tumor necrosis factor, and delayed-type hypersensitivity. The incubation of chlorpromazine in the concentration of 10 μg/ml slightly decreased TNF-α from about 3500–3250 pg/ml, while in the case of 10 H-2,7-diazaphenothiazine the decrease was from about 3500–1500 pg/ml (Zimecki et al., 2009).

Li et al. (2020) analyzed the effect of trifluoperazine (30 μM) on necrosis of multiple myeloma U266 and RPMI 8226 cells. The authors used LDH release assay and PI flow cytometry and annexin V staining. In the case of LDH release, no significant differences were observed suggesting that trifluoperazine did not lead to necrosis in myeloma cells. The flow cytometry assay confirmed previous observations since the

**Table 1**
The effect of phenothiazine derivatives on the necrosis process.

| Phenothiazine derivatives | Localization and the used cell line | Necrosis impact | References |
|---|---|---|---|
| **Chlorpromazine** | Circulatory system: human whole blood culture | inhibition: ↓ TNF-α | (Zimecki et al., 2009) |
| | Immune system: Jurkat cells and rat peripheral blood mononuclear cells (PMBC) | induction: ↑ in necrotic cells number | (Wolnicka-Głubisz et al., 2002) |
| | Liver: hepatocytes and Kupffer cells of healthy male pigs | induction: ↑ TNF-α | (Hoebe et al., 2000) |
| | Skin: HaCaT cells | induction: ↑ in necrotic cells number | (Kurita et al., 2007) |
| | *In vivo*: male $B_6D_2F_1$ mice | inhibition: ↓ TNF-α | (Clancy et al., 2000) |
| **Fluphenazine** | Brain: isolated crayfish neuron and satellite glial cells | induction: spontaneous necrosis of stretch receptor neuron and glial cells, % of necrotic cells: about 30%, 20%, 75%, and 40%, respectively for the control, fluphenazine (1 μM), PDT, as well as fluphenazine (1 μM) and PDT | (Uzdensky et al., 2007) |
| **Levomepromazine** | Breast: MCF-7 | induction: ↑ in early and late necrotic cells number | (Seo and Efferth, 2016) |
| **Perphenazine** | Brain: human neuroblastoma SH-SY5Y | induction: ↓ ATP level about 80% and 40% after 8 and 16 h treatment | (Hong et al., 2012) |
| | Immune system: human Sezary syndrome cell line (HUT78) | induction: about 30% late apoptotic/necrotic cells after perphenazine treatment | (Tsuji et al., 2021) |
| | Skin: UACC 903 cells | induction: ↑ in early and late necrotic cells number | (Kuzu et al., 2017) |
| **Promethazine** | *In vivo*: 43- and 26-year-old women | induction: intra-arterial administration of promethazine is associated with tissue necrosis | (Foret et al., 2009) |
| **Thioridazine** | Brain: human glioblastoma U-87 MG and GBM8401 | induction: ↑ number of late apoptotic or necrotic cells | (Chu et al., 2019) |
| | Sex organs: SiHa cells | induction: ↑ HMGB1 | (Mao et al., 2015) |
| **Trifluoperazine** | Brain: human neuroblastoma SH-SY5Y | induction: ↓ ATP levels 90% and 60% for trifluoperazine 8 and 16 h-treatment | (Hong et al., 2012) |
| | Hematopoietic system: U266 and RPMI 8226 cells | No impact: LDH release similar to the control, flow cytometry with annexin V and PI staining suggests apoptosis | (Li et al., 2020) |
| | Pancreas: MiaPaCa-2 cells | induction: dose-dependent ↑ of PI-positive cells, ↑ in LDH release, ↓ ATP level | (Huang et al., 2019) |
| | Sex organs: HeLa cells | induction: ↑ in early and late necrotic cells number | (Menilli et al., 2019) |
| **Novel phenothiazine derivative (DS00329)** | Brain: human glioblastoma U251 and U-87 MG | Low induction/ no impact: ↑ the number of necrotic cells (U-251) or the same number of necrotic cells as in the control (U-87 MG) | (Omoruyi et al., 2020) |
| **Fluphenazine and fluphenazine derivatives (compounds 3–12)** | Immune system: human lymphocytes genotoxically damaged by incubation with B[*a*]P | induction: compounds 3, 9, and 10 caused the weakest influence on the frequency of necrotic lymphocytes, the apoptosis/necrosis index about 10–15 times lower than for fluphenazine and compounds 5, 10, and 11. | (Zyta et al., 2014) |
| **Trifluoperazine (ZZW-111) and six trifluoperazine derivatives (ZZW-112, ZZW-115, ZZW-116, ZZW-119, ZZW-120, and ZZW-124)** | Pancreas: MiaPaCa-2 (a traditional cell line), 02.063 and LIPC (basal subtype), Foie8b (derived from a liver metastasis), and HN14 (classical subtype) | induction: ↑ in necrotic cells, LDH release | (Santofimia-Castaño et al., 2019) |
| **10 H-2,7-diazaphenothiazine** | Circulatory system: human whole blood culture | inhibition: ↓ TNF-α | (Zimecki et al., 2009) |

apoptotic rate was higher in the trifluoperazine group than in the control (Li et al., 2020).

Hoebe et al. (2000) measured TNF-α in hepatocytes and Kupffer cells of healthy male pigs after chlorpromazine treatment. The authors did not record any crucial rise in TNF-α production after incubation of the cells with different concentrations of chlorpromazine (Hoebe et al., 2000).

### 3.2. *In vivo studies*

Clancy et al. (2000) analyzed the level of TNF-α in male $B_6D_2F_1$ mice which were given a 4 mg/kg i.p. dose of chlorpromazine hydrochloride 1 h before endotoxin administration. The authors observed that significant decrease in TNF-α mRNA levels to baseline levels in the liver and lungs at 40 min, 1 h, and 2 h after endotoxin was administered in the chlorpromazine-treated group. TNF-α expression was significantly increased by chlorpromazine pretreatment, 24 h after endotoxin treatment. For the chlorpromazine-treated mice, there was a significant reduction in liver TNF-α protein levels at 1 and 2 h. Interestingly, the recorded change in TNF-α protein corresponds to the decrease in TNF-α transcript levels (Clancy et al., 2000).

Foret et al. (2009) observed two cases of necrosis caused by promethazine injected intra-arterially. The first case was a 43-year-old woman who was to be injected with promethazine into the left antecubital IV line. Unfortunately, by accident, promethazine was administered into the brachial artery of the left arm. After 5 days the patient returned to the emergency with pain and discoloration of the left index and ring fingers. Moreover, her thumb and little finger turned purple. At another emergency department, the vascular surgeon observed that there was a purplish discoloration of the digits of the left hand. After 6 weeks necrosis developed in all digits of the patient's left hand. The second case was a 26-year-old woman who was infused (normal saline, 50 mg meperidine, and 12.5 mg promethazine) *via* a newly placed 24-gauge angiocatheter. Demarcation of the terminal segment of the left index finger occurred after two weeks, whereas the left index finger had fully demarcated from the middle segment distally after three weeks of the initial infusion. This necessitated amputation, which indicates that the intra-arterial administration of promethazine can lead to tissue necrosis (Foret et al., 2009).

We prepared Table 1 to offer a concise representation the effect of phenothiazine derivatives on necrosis in different cell lines.

The above table shows how phenothiazine derivatives can mainly induce the necroptosis and necrosis, however the used cell line affects the processes, which may be explained by different response of cells due to their different biological properties. Chlorpromazine induces necrosis in the immune system and liver cell lines, while in human whole blood

cells and *in vivo* studies chlorpromazine decreases the level of TNF-α, which suggests disturbances/decrease of necrosis. Fluphenazine and novel derivative DS00329 induce necrosis in brain cell lines. Levomepromazine induces necrosis in breast cell lines only, while perphenazine induces necrosis in the brain, the immune system, and skin cell lines. Thioridazine induces necrosis in the brain and sex organ cells, while trifluoperazine additionally induces the process in pancreas cells. Interestingly, trifluoperazine does not induce necrosis in hemopoietic system cells. This may be explained not only by the different types of cells, but also by the concentration of the used drug *e.g.* the groups analyzing trifluoperazine in the concentration up to 25 μM observed necrosis and apoptosis, while the group using the concentration of 30 μM did not confirm any necrosis, but apoptosis only. Fluphenazine and fluphenazine derivatives (compounds 3–12) induce necrosis in the immune system cells only, while trifluoperazine and trifluoperazine derivatives induce necrosis in pancreas cell lines. It is worth observing that the *in vivo* study using promethazine confirmed also induction of necrosis after intra-arterial administration.

## 4. The molecular mechanism of necrosis and necroptosis

Necroptosis starts when the tumor necrosis factor (TNF) family of cytokines such as TNF-α have been activated (Wang et al., 2022; Wu et al., 2020), TNF-related apoptosis-inducing ligand (Wu et al., 2020), factor-associated suicide ligand (FASL) (Wang et al., 2022; Wu et al., 2020), lipopolysaccharide (LPS) (Wang et al., 2022; Wu et al., 2020), double-stranded RNA (dsRNA) (Wang et al., 2022; Wu et al., 2020), viral double-stranded DNA (dsDNA) (Wu et al., 2020), interferon-γ (IFN- γ) (Wang et al., 2022; Wu et al., 2020), or TNF-related apoptosis-inducing ligand (TRAIL) (Wang et al., 2022), which also regulates cell survival and apoptosis induction (Tan et al., 2018). Other mediators of necroptosis are: calcium (Vanden Berghe et al., 2010), ceramide (Vanden Berghe et al., 2010), cytosolic phospholipase A2 (cPLA2) (Vanden Berghe et al., 2010), lysosomal membrane permeabilization (LMP) (Vanden Berghe et al., 2010), and N-methyl-N0-nitro-N-nitrosoguanidine (related to the expression of poly ADP-ribose polymerase (PARP)) (Wu et al., 2020). Whereas, necrosis is $H_2O_2$ or Fas induced (Vanden Berghe et al., 2010). Binding of TNF-α to the membrane receptor TNFR1 promotes the formation of a plasma membrane-associated complex I consisting of adaptor proteins tumor necrosis factor receptor 1 (TNFR1)-associated death domain protein (TRADD), receptor-interacting serine-threonine kinase 1 (RIPK1), TNFR-associated factor 2/5 (TRAF2/5), a cellular inhibitor of apoptosis protein 1/2 (cIAP1/2) and the linear ubiquitin chain assembly complex (LUBAC complex) (Ketelut-Carneiro and Fitzgerald, 2022; Wang et al., 2022; Wu et al., 2020). TRAF ubiquitinates and stabilizes RIPK1 at the plasma membrane, FADD recruits pro-caspase-8, and RIPK1 mobilizes RIPK3 (Ketelut-Carneiro and Fitzgerald, 2022).

At this point, the cell possesses three options. Firstly, if there is ubiquitin-mediated regulation of RIPK1 (by cIAPs and TRAF2/5) cell death does not occur (Ketelut-Carneiro and Fitzgerald, 2022). Polyubiquitinated RIPK1 can activate the NF-kB pathway and mitogen-activated protein kinases (MAPKs) (Wang et al., 2022; Wu et al., 2020). It is worth observing that the Iκβ kinase (IKKα/β) is recruited by a nuclear factor-kappa β essential modulator (NEMO, also known as IKKγ) into complex I, which phosphorylates RIPK1 at Ser25 leading to inhibition of RIPK1 kinase-dependent cell death (Xu and Huang, 2022). Secondly, in the case of cIAP inhibition and deubiquitination of RIPK1 by cylindromatosis lysine 63 deubiquitinase (CYLD) (Ketelut-Carneiro and Fitzgerald, 2022; Wang et al., 2022) or if RIPK3 is absent or it is present at a low level (Ketelut-Carneiro and Fitzgerald, 2022), RIPK1 and TRADD dissociate from complex I (Ketelut-Carneiro and Fitzgerald, 2022; Wang et al., 2022; Wu et al., 2020). Next, a complex called cytosolic death-inducing signaling complex (DISC) is formed by binding RIPK1 and TRADD to procaspase-8 and Fas-associated death domain (FADD) (Wang et al., 2022; Wu et al., 2020). FADD comes from interaction FAS with FASL as well as TRAIL with DR4/5, which led to the binding of FADD by the intracellular DD domain and finally led to form the DISC (Wu et al., 2020). DISC, called also complex Ia (Wang et al., 2022), can initiate caspase 8 and apoptosis as well as inhibit necrosis (by cleaving its core regulators, RIPK1 and RIPK3) (Ketelut-Carneiro and Fitzgerald, 2022; Tan et al., 2018; Wu et al., 2020). The cellular FLIP long isoform protein (cFLIPL) (Wu et al., 2020), pan-caspase inhibitor (zVAD), or FLICE inhibitory protein (FLIP) (Wang et al., 2022) may inhibit the activation of caspase 8 Thirdly, when caspase-8 activity is inhibited (Ketelut-Carneiro and Fitzgerald, 2022) or RIPK3 overexpressed (Wu et al., 2020) RIPK3 and RIPK1 bind to DISC to form the necrosome complex, which promotes necroptosis (Ketelut-Carneiro and Fitzgerald, 2022; Wu et al., 2020). Necroptosis is executed when the apoptosis is blocked (Tan et al., 2018; Vanden Berghe et al., 2010). The activated receptor interacts with receptor-interacting serine/threonine-protein kinase (RIPK) 1 and recruits inhibitors of apoptosis: Baculovirus Inhibitor of apoptosis protein Repeat domain (BIRC) 2 and 3, which leads to forming a membrane-associated complex that regulates cell survival through NF-kB and mitogen-activated protein kinases (MAPKs) pathways (Tan et al., 2018). Another, well-known inhibitor of necrosis is Nec-1, which can reverse cell death. The inducers and inhibitors of necroptosis are very well described by Wu et al. (2020).

The detailed process of necrosome forming includes binding of RIPK 1 to RIPK3 and forming RIP homotypic interaction motif (RHIM) (Wang et al., 2022; Wu et al., 2020) insoluble amyloid complex called necrosome (Tan et al., 2018). For the stabilization of the necrosome complex, RIPK3 must ubiquitinate Lys5 of RIPK3 (Seo et al., 2021). The process of forming a necrosome promotes autophosphorylation of RIPK1 and RIPK3, which in turn recruits and phosphorylates the pseudokinase, mixed lineage-like kinase (MLKL) (Tan et al., 2018). The autophosphorylation sites of RIPK1 are: Ser14/15, Ser20, Ser161, and Ser166 (Xu and Huang, 2022). The autophosphorylation process of RIPK1 is important for its enzymatic activation (Xu and Huang, 2022). The autophosphorylation Ser161 on RIPK1 is promoted by ROS (Liang et al., 2021). Autophosphorylated by casein kinase 1 (CK1) family, serine/threonine kinases, Ser227 of RIPK3 forms a hydrogen bond with Ser404 of MLKL resulting in MLKL phosphorylation and necroptosis induction (Seo et al., 2021; Xu and Huang, 2022). It should be emphasized that the loss of CK1 results in a decrease in RIPK3 phosphorylation at Ser227, which indicates its important role in necroptosis promotion (Seo et al., 2021). Interestingly, phosphorylation Ser345 is not important for binding RIPK3 to MLKL, but for MLKL translocation, accumulation in the plasma membrane, and subsequent necroptosis (Xu and Huang, 2022).

Next, the MLKL monomer is phosphorylated (at the Thr357 and Ser358 in the pseudokinase domain (Seo et al., 2021; Xu and Huang, 2022)), which leads to MLKL oligomerization (by a conformational change in the 4HB domain (Xu and Huang, 2022)) to form a trimer, inserted into the membrane to form a pore, which leads to necroptosis because plasma and intracellular membrane integrity are lost (Tan et al., 2018), and there is influx of calcium ions (Wang et al., 2022). TYRO3, AXL, and MER receptor kinases (TAMs) as MLKL kinase phosphorylate Tyr376 of MLKL promoting its oligomerization (Seo et al., 2021; Xu and Huang, 2022). MLKL trimers bind to phosphatidylinositol phosphates (PIPs) and cardiolipin (CL), which are mitochondria-specific. This leads to their translocation to the membrane rich in PIPs and CL (Wu et al., 2020). In consequence, mitochondrial dysfunction occurs, reactive oxygen species (ROS) are excessively produced, and the following are observed: ATP depletion, cell plasma membrane damage, cell content spillage, and swelling of cells and organelles (Wang et al., 2022). Necrosis is morphologically characterized by "rounding of the cell, cytoplasmic swelling, presence of dilated organelles and absence of chromatin condensation" (Vanden Berghe et al., 2010). Since the cellular membrane is damaged, necrosis may be detected by the release of lactate dehydrogenase (LDH). When the plasma membrane is damaged, LDH is released into the extracellular space (Chan et al.,

2013). Moreover, necrotic cells passively release nuclear high-mobility group box 1 (HMGB1) which results in an inflammatory reaction *via* TNF-α (Chen et al., 2022; Xue et al., 2021).

Moreover, the activation of TLR3 by binding dsDNA or TLR4 by binding LPS activates TIR-domain-containing adapter-inducing interferon-β (TRIF) to finally form TRIF-RIPK3-MLKL necrosome (RIPK3 comprising RHIM protein domains) and induce RIPK3-dependent necroptosis (Wang et al., 2022; Wu et al., 2020) or TRIF stimulates RIPK3 interaction to form homo-dimer, which is autophosphorylated and forms a complex with MLKL (Ketelut-Carneiro and Fitzgerald, 2022). RIPK1-independent necroptosis is possible after a viral infection when DNA of a virus activates the DNA-dependent activator of the interferon regulatory factors (DAI) pathway, which leads to interaction with RIP3 *via* RHIM domain. It also activates interferon regulatory factor 3 (IRF3) and NF-κB pathways, and cytokines and type1 interferon responses in turn (Wang et al., 2022; Wu et al., 2020). It is worth noting that a marked depletion of cellular ATP can be observed in necroptotic cells (Chen et al., 2016).

We prepared Fig. 2 to summarize the information on the necroptosis mechanism, and to show how it can be potentially regulated by phenothiazine derivatives.

## 5. Conclusions

Based on the analyzed results of *in vivo* and *in vitro* research, which focused on the impact of chlorpromazine, fluphenazine, levomepromazine, perphenazine, promethazine, thioridazine, trifluoperazine, and novel derivatives on necrosis and necroptosis, it can be concluded that phenothiazine derivatives are able to modulate both processes. However, various factors determine whether either necrosis or necroptosis will occur, and they are the cell line (different types of cells can respond differently to the same stimuli), the type of analyzed phenothiazine (phenothiazines differ regarding the strength of interaction and relation to the receptors), and the used concentration. Unfortunately, it is not possible to present a mechanism of action, because in all the analyzed papers the obtained results were based on TNF-α and ATP levels as well as the final steps of necrosis/necroptosis related to membrane permeability (PI staining, LDH release, and HMGB1 amount). It is worth noting

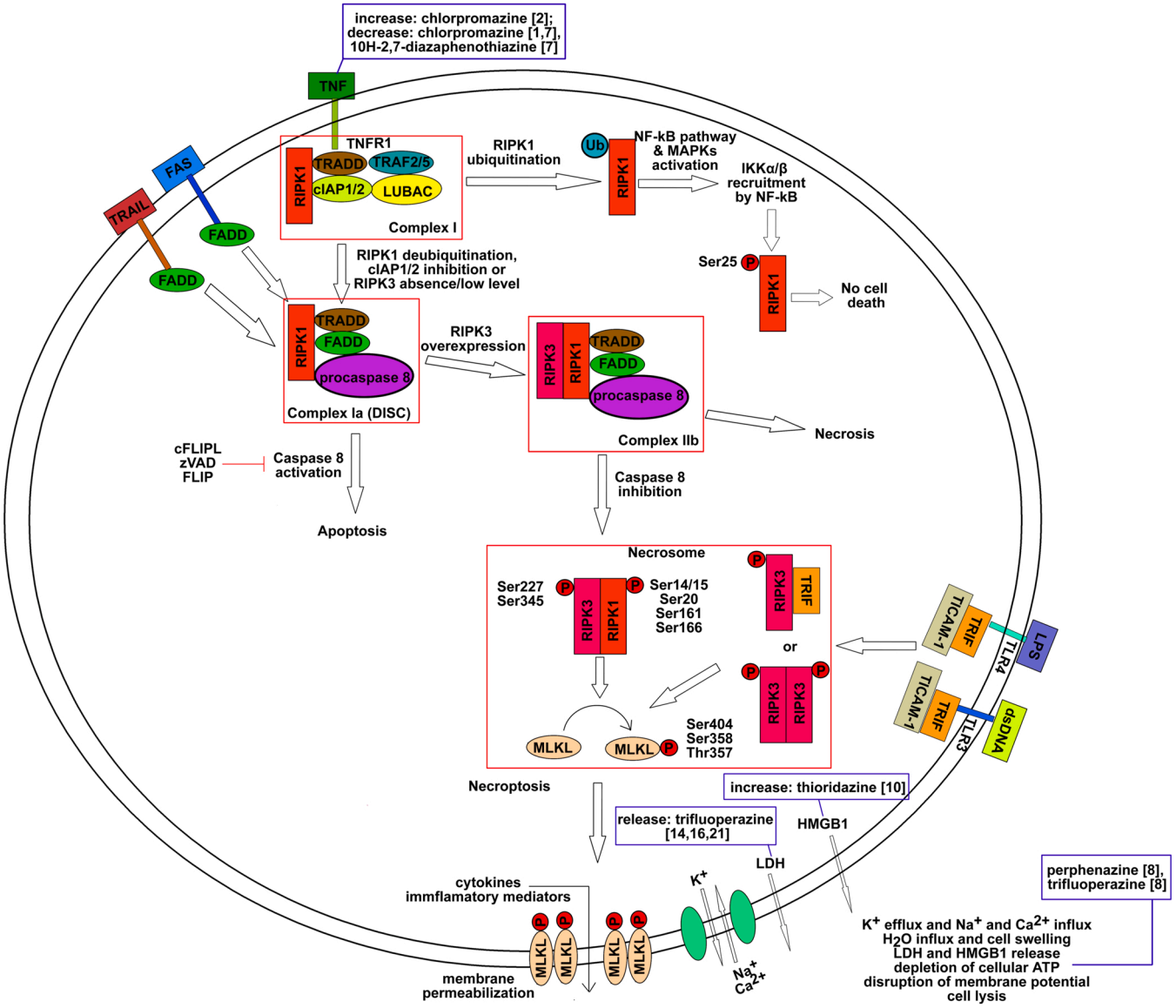


**Fig. 2.** The mechanism of necroptosis and phenothiazine derivatives potential points of action in the process (Chen et al., 2016, 2022; Ketelut-Carneiro and Fitzgerald, 2022; Liang et al., 2021; Seo at al., 2021; Tan et al., 2018; Vanden Berghe et al., 2010; Wang et al., 2022; Wu et al., 2020; Xu and huang, 2022; Xue et al., 2021).

that the flow cytometry assay, with common stains such as annexin V, PI or 7-aminoactinomycin D (7-AAD) used to differentiate apoptotic and necrotic cells, can generate several misinterpretation problems for non-specialists in cell biology. In apoptotic cells, or cells undergoing the apoptotic process, phosphatidylserine is externalized giving a signal to phagocytic systems to eliminate the cells. However, in *in vitro* experiment a chemical substance or another stimulus can trigger cell death in tumor or non-tumor cells. This usually leads to loss of the integrity of their plasma membrane and to secondary necrosis (as an artifact) instead of phagocytosis. Thus, other assays should be used concomitantly to exclude or confirm the occurrence of necrosis (or necroptosis).

Most of the presented results suggest necroptosis induction, only a few reports mention inhibition, low induction/no impact of novel derivative DS00329 on necroptosis of human brain cancer cells, and no impact of trifluoperazine on hematopoietic system cells. Moreover, we did not find any papers about the impact of phenothiazine derivatives on RIPK1, RIPK3, or MLKL, which are key molecules of necroptosis.

It is important to analyze the expression of those proteins as well as use necrosis (Nec-1, IM-54, Necrostatin-5) and necroptosis (Necrostatin-1) inhibitors since only then the effect of phenothiazine derivatives on necroptosis and necrosis can be understood. Moreover, it will not be possible to comprehend the mechanism of action of phenothiazine derivatives and introduce them into cancer therapy without thorough research.

## CRediT authorship contribution statement

Otręba M: Conceptualization, Methodology, Data analysis, Results interpretation, Visulaization and Writing –Original Darft preparation. Stojko J: Original Darft Preparation. Rzepecka-Stojko A: Supervision, Writing-Revieving.

## Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Michal Otreba reports financial support was provided by Medical University of Silesia. Anna Rzepecka-Stojko reports financial support was provided by Medical University of Silesia.

## Data Availability

No data was used for the research described in the article.

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### *Declarations*

None.

### *Conflict of interest*

The authors declare that they have no conflict of interest.

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