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Review

The Anticancer Activity of the Old Neuroleptic Phenothiazine-type Drug Thioridazine

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Abstract. *Thioridazine (TZ), an antipsychotic drug, renders multidrug-resistant (MDR) cancer cells susceptible to cytotoxic agents to which they were initially resistant, has anti-prolilferative activity and apoptosis-inducing properties in various tumor cell lines and cancer stem cells. Whereas the anti-proliferative activity takes place at high concentrations that ensure the intercalation of the compound between nucleic bases (especially rich in G/C bases), much lower concentrations inhibit the export function of the ABCB1 (P-glycoprotein), which is responsible for the MDR phenotype of the cancer cell. The co-administration of TZ with doxorubicin inhibits efflux of doxorubicin and, hence, increases the intracellular concentration of anticancer drug. The (+) and (–) enantiomers of TZ have the same activities as TZ. The main focus of this review is to present extensive evidence provided by our work, confirmed by much later studies, as it supports adjuvant use of TZ with an anticancer drug for MDR cancer therapy.*

Phenothiazines are heterocyclic compounds that have specific and non-specific activities against constituents of the plasma membrane of eukaryotes. Although the first phenothiazines were synthesized in the late 19th century, the basic phenothiazine structure is identical to that of the well-known dye methylene blue, which was first prepared as a stain in 1876 by the German chemist Heinrich Caro as a derivative from an existing phenothiazine (1). Paul Ehrlich studied methylene blue (Figure 1) for biological properties and found that it could reduce the mobility of bacteria (1). The demonstration by Bodoni at the end of the 19th century that methylene blue could render a human lethargic, spurred investigations of the possible use of this dye to control psychosis (2). It took more than 50 years to produce a colorless methylene blue-type compound by the French chemist Charpentier of Rhone-Poulenc called chlorpromazine (CPZ) that had the desired neuroleptic properties for therapy psychosis (3). However, because of its toxic side-effects, the compound was soon replaced with the equally effective phenothiazine neuroleptic thioridazine (TZ) whose most frequent side-effect was somnolence (Figure 1). The early wide use of CPZ indicated that, apart from their basic neuroleptic action related to the blockade of dopaminergic receptors, CPZ affected diverse biological activities, such as the calcium transporter calmodulin, protein kinase C, as well as many other enzymes that, when inhibited by CPZ, promoted anti-proliferative effects (4, 5). The enhancement of antibiotic activity by CPZ on bacteria and the demonstration that multidrug-resistant (MDR) *Mycobacterium tuberculosis* could be inhibited in its replication by CPZ and TZ (6) as well as the reversal of antibiotic resistance was the result of inhibition of the efflux pump system of mycobacteria (6) and bacteria (7). In addition, similar inhibitory effects of the ABCB1 transport function were demonstrated using MDR mouse T-lymphoma cells (8). The relevant possibilities to MDR cancer were supported by the anti-helmintic and antibacterial activities of phenothiazines described in the 1930s and 1940s (5). Phenothiazines have been in use for the treatment of psychiatric disorders since the 1950s. In addition, various other effects of phenothiazines have since been described,

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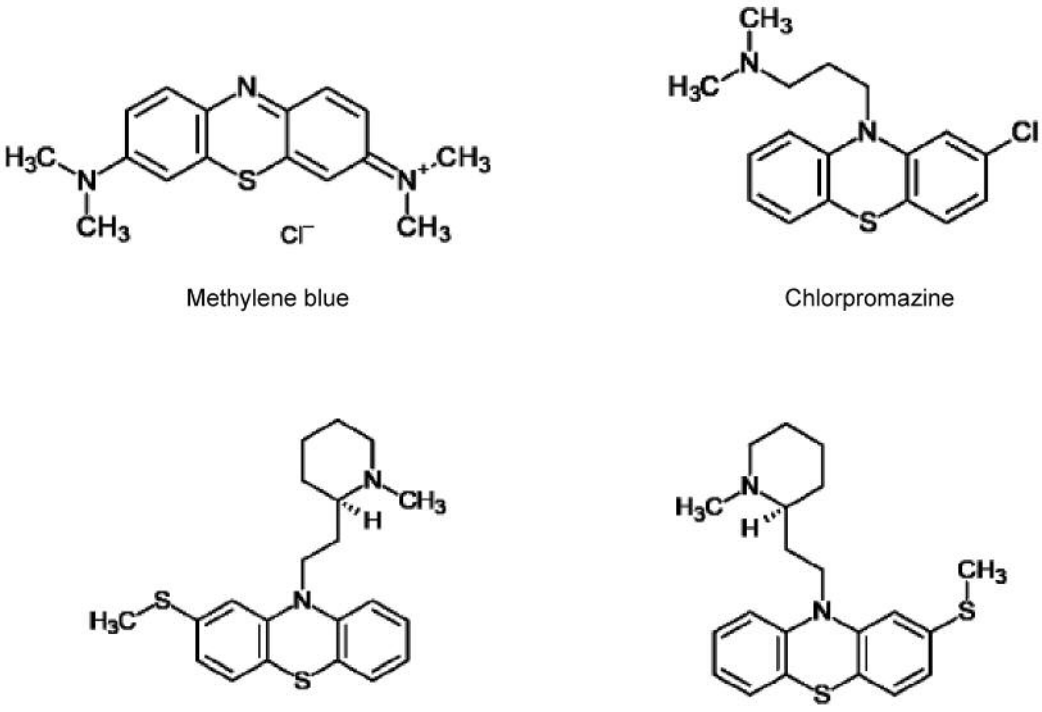

Figure 1. *Chemical structure of some important phenothiazines.*

such as anti-mycobacterial (9), anti-plasmid (10) and immunomodulatory (11) activities. The substituents in the phenothiazine ring, which increase lipophilicity, amplify their anti-proliferative activity in cancer cells (12). The presence of different substituents into the phenothiazine skeleton, as well as the modification of the tricyclic ring system, alters their biological effect (13). Because of their highlighted biological activity, numerous derivatives of phenothiazines have been synthesized and patented (5).

Thioridazine, (10-[2-(1-methyl-2-piperidinyl)ethyl]-2-(methylthio)-10H-phenothiazine), was previously used extensively as an antipsychotic drug; however, there is an increasing number of studies demonstrating its anticancer, multidrug resistance-reversing and apoptosis-inducing properties in various tumor cell lines. This review intends to give an overview of the MDR-reversing activity of thioridazine that has resulted from our decades of published research studies, many of which have been recently confirmed by others, although correct citation of their existence has been omitted willfully or otherwise.

Membrane Destabilizing Effects

Phenothiazines demonstrate a strong affinity to lipid bilayers of cell membranes because of the high degree of lipophilicity of the phenothiazine ring (12). Thioridazine (TZ) interacts with the inner mitochondrial membrane. Consequently, TZ presents with antioxidant and peroxidation activities on membrane lipids of the mitochondria, with these effects resulting in transient alterations of mitochondrial permeability, thus promoting release of cytochrome *c* from mitochondria (14).

Effect on Cell Cycle

According to *in silico* gene signature based studies, TZ inhibits the phosphatidylinositol-3'-kinase (PI3K)/Akt pathway and, thus, exerts cytotoxicity in ovarian cancer cells (15).

Translationally controlled tumor protein (TCTP) is an essential regulator of apoptosis and cell cycle events (16). In 508 breast cancers, high-TCTP status is associated with aggressive G3-grade tumors, predicting poor prognosis. TZ increases P53 expression by counteracting the ubiquitination of P53 that is enhanced by TCTP (17).

Anti-calmodulin Activity

Calmodulin is a calcium binding protein that regulates calcium-dependent biochemical processes and, hence is involved in a large variety of cellular functions. Since TZ is a potent inhibitor of calmodulin, calcium binding and many calcium-dependent enzymes that are involved in cellular proliferation become destabilized. Thus, when calmodulin is

inhibited from binding calcium by TZ, these enzymes deny access to calcium, and, hence, their reduced activity results in the inhibition of cell proliferation (12). Similarly, many biochemical pathways, dependent upon access to calcium, are similarly inhibited (12, 18).

Effect on DNA Damage

TZ can interfere with DNA damage responses and DNA repair. Furthermore, TZ is an inhibitor of Tousled-like kinases (TLKs), enzymes responsible for the maintenance of genomic stability (19). As described previously, TZ has anti-plasmid activity by eliminating the drug resistance-carrying plasmids of bacteria (20).

TZ as Dopamine Receptor Antagonist

TZ is an antagonist of the dopamine D2 receptor (DRD2) and, according to previous reports, DRD2 could be a promising therapeutic target in cervical cancer therapy. It has been shown that TZ inhibits cellular proliferation through down-regulation of DRD2 expression. Moreover, TZ can inhibit proliferation of human uterine cervical carcinoma cells and induce apoptosis and necrosis (21). TZ can inhibit the PI3K/Akt/mTOR/p70S6K signaling pathway and has cytotoxic activity on cervical and endometrial cancer cells by inducing cell cycle arrest and apoptosis (22).

Activity on Stem Cells

TZ is a selective inducer of the differentiation of cancer stem cells (CSC) and the anti-CSC activity of TZ is due to its antagonistic effect on DRD2 receptors that are differentially expressed on neoplastic stem cells. It is important to note that TZ has no effect on normal human somatic stem cells implying that TZ could be a selective anti-CSC drug (23).

TZ exerts an apoptotic effect in brain-derived tumors; furthermore, the sensitivity of neoplasm tissue to thioridazine is higher than that on primary (normal) brain cells (24). With the use of bioinformatic tools, it has been demonstrated that TZ is a potent anti-glioblastoma and anti-glioblastoma cancer stem-like cell agent. Moreover, because autophagy might be a major mechanism of the anti-glioblastoma activity and TZ has proved to be an effective glioblastoma tumor growth inhibitor that can induce autophagy *in vivo*, the rediscovery of TZ as an anticancer agent could be used as a powerful strategy against malignant tumors with a poor prognosis (25).

Apoptosis Induction

TZ induces apoptosis in leukemic cells without any influence on the viability of normal lymphocytes (26). It also induces apoptosis of B16 melanoma cells and demonstrates *in vivo* anti-tumor activity (27). The *in vivo* activity of TZ was studied using female C57/Bl mice. Animals were inoculated with wild-type B16 melanoma cells by intravenous (*i.v.*) injection into the tail vein. Mice were treated with TZ (10 and 15 mg/kg × 3/weeks intraperitoneally (*i.p.*) or 15 and 25 mg/kg/day *per os* (*p.o.*)) and, after autopsy, the lung weight and number of pulmonary melanoma colonies were determined. TZ administration (*i.p.* or *p.o.*) resulted in the reduction of lung tumor burden and an increase in mice survival (27).

It was demonstrated that TZ induces apoptosis of the MDR mouse T-lymphoma cells at 5 and 10 μg/ml concentrations (8). During apoptosis, the plasma membrane undergoes multiple changes: the translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane can be detected by annexin V. After treatment with 10 μg/ml of TZ, 49.54% of the cells were early-apoptotic and 27.26% showed late-apoptotic and necrotic features, although the proportion of dead cells was 3.4% in MDR mouse T-lymphoma cells (8). The apoptosis-inducing effect of TZ was confirmed by other studies using various cell lines (28).

Efflux Pump Inhibition

The major mechanism for the MDR phenotype is the overexpression of ATP-dependent transporters known as the ATP-binding cassette (ABC) family. In humans, the three major types of MDR proteins include members of the ABCB (ABCB1/MDR1/P-glycoprotein), the ABCC (ABCC1/MRP1, ABCC2/MRP2, probably also ABCC3–6 and ABCC10–11) and the ABCG (ABCG2/MXR/BCRP) sub-family (29). The relationship between ABC expression levels and sensitivity to drugs or possible drug candidates is of great importance concerning anticancer chemotherapy.

Substances, such as verapamil and TZ, which can block ABCB1 or P-glycoprotein, can reverse resistance of doxorubicin-resistant sarcoma 180 (S180) cells completely (30). According to Spengler *et al*., TZ induces apoptosis of the MDR lymphoma cells and inhibits the activity of their overexpressed ABCB1 transporter. However, the concentrations that produce these *in vitro* results are extremely high and greater than that clinically employed for the therapy of severe psychosis (8).

Anti-angiogenesis

Angiogenesis is essential for tumor growth and metastasis and targeting angiogenesis could be a promising objective for anticancer drug development. Expression of vascular endothelial growth factor (VEGF) and hypoxia-inducible factor 1α (HIF-1α), as well as the phosphorylation of VEGFR-2 were reduced in a thioridazine-treated tumor (31).

TZ is an angiostatic agent found to effectively inhibit angiogenesis in a murine tumor model reducing microvessel density *in vivo* (32).

Stereochemistry and Pharmacological Activity

The consideration of stereochemistry in drug action is gaining ever-greater attention in medical practice. Many of the drugs currently used in psychiatric practice are mixtures of enantiomers. For some therapeutics, single-enantiomer formulations can provide greater selectivity for their biological targets and/or better pharmacokinetics compared to the mixture of enantiomers. When both a single enantiomer and a racemic formulation of a drug are available, the information from experimental evidences should be used to decide which formulation is most appropriate (33). The potential advantages of using single enantiomers of a chiral drug were investigated by Csonka *et al*. (34). The equilibrium between the (+) and (–) isomers influences the binding affinities of the enantiomers to different ligands or receptors. "Do the racemate TZ and its enantiomers express different activities against cancer cell lines?" This question was based upon observations published by others who suggested that "the (–)-TZ enantiomer had slightly more catalepsy (neuroleptic)" (35). Nevertheless, a variety of pharmacological studies, as reported by Svendsen *et al*. (35), indicate significantly greater binding of the (–)-TZ enantiomer to the D1 receptor of the rodent brain. Regardless, our results clearly demonstrate that the racemate TZ and its enantiomers have essentially the same activity against cancer cell lines with respect to the inhibition of replication, induction of apoptosis and inhibition of ABCB1 (34). The central nervous system (CNS) receptor stereospecificity of (+) and (–)-TZ was first described by Svendsen *et al*. in 1988 (35). Optical isomers, such as those of TZ, exhibit significant differences in their affinities for receptor sites, biotransformation and binding to serum and tissue proteins. Separation of the racemate TZ into its enantiomers in the human body has been measured (36). In these investigations, the (–) enantiomer was found to be at higher concentrations than the (+) enantiomer in the different tissues. The general possibility for using the resolution of commercially available racemates in therapeutics for an anxiolytic effect has been described by Baumann *et al*. (36) based on the investigations and statements made by Ariens *et al*. (37). The role of the chirality of other compounds known to affect ABCB1, such as the enantiomers of verapamil, has been investigated. These latter studies indicated that the L and the D forms of verapamil had equal abilities to reverse the *in vitro* resistance of MDR leukemic cells to drugs, such as vincristine (38). The mechanism of action of TZ has been analyzed in detail by Spengler *et al*. who showed that the racemate is able to induce the apoptosis of MDR mouse T-lymphoma cells (8). In this latter study, the differential effects of the two TZ enantiomers were not examined. As previously stated, the anti-proliferative effects of the racemate and (+) and (–) enantiomers of TZ on MDR mouse T-lymphoma cells overexpressing ABCB1 were investigated by Csonka *et al*. (34) and no significant differences were found between the three compounds.

In order to avoid the toxic side-effect of resistance-reversing compounds, drugs with selective inhibition of the MDR in cancer cells are needed. To achieve this effect, three classes of known neuroleptic drugs (methotrimeprazine, clopenthixol and butaclamol isomers) with active and inactive stereoisomers were tested for MDR efflux pump inhibition on mouse T-lymphoma cell lines (39). Their anti-proliferative effects on sensitive and MDR cancer cell cultures were compared with those of the classical resistance modifier verapamil. It was confirmed that enantiomers of phenylalkylamines proved to be equally potent inhibitors of drug transport by ABCB1 (39). However, CNS-active and -inactive butaclamol enantiomers exerted slightly different effects on the reversal of the MDR phenotype, which means that drug binding might have weak enantioselectivity for ABCB1 (39), suggesting that some enantiomers of compounds that inhibit ABCB1 can be exploited as adjuvants to cytostatic chemotherapy of cancer in order to increase the efficacy of the anticancer agent. However, the separate use of one enantiomer of TZ *versus* the other did not appear to provide any advantage over racemic TZ for the adjuvant therapy of MDR cancer.

Role of TZ in Combined Chemotherapy

The combination therapy using a conventional chemotherapeutic drug with an agent that can improve the action of the anticancer drug without any additional side-effects could be a good approach to treat MDR cancer. TZ showed a moderate synergism to doxorubicin in P388/ADR murine leukemia cells described by Ramu *et al*. (40).

It has been confirmed that calmodulin inhibitors significantly enhanced the cytotoxic effects of doxorubicin in the resistant but not parent-sensitive P388 cells (41). To administer TZ and doxorubicin to *in vitro* and *in vivo* systems, polymeric micelles have been designed as delivery carriers (42). The combination therapy with TZ-free or TZ-loaded micelles was effective in decreasing the population of cancer stem cells. However, TZ-loaded micelles had lower antitumor efficacy than doxorubicin-loaded micelles; the combination therapy of TZ-loaded and doxorubicin-loaded micelles presented a greater antitumor effect than doxorubicin-loaded micelles alone (42).

Conclusion

The first demonstrations that phenothiazines could inhibit cancer growth were reported during the early 1950s. Since those early years, many studies have been published

describing that phenothiazines can inhibit the growth of some types of cancers (43). Based on the biological activity of TZ, such as interference with membrane function, DNA repair, signaling pathways, cell cycle, apoptosis induction, efflux inhibition and, also, its synergistic effect with doxorubicin, renders TZ a powerful anticancer drug and adjuvant in combined chemotherapy.

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