One of the systems responsible for maintaining cellular redox homeostasis is the thioredoxin-dependent system. An equally important function of this system is the regulation of the expression of many proteins by the transcription factor NF-κB or the apoptosis regulating kinase (ASK-1). Since it has been shown that the Trx-dependent system can contribute to both the enhancement of tumour angiogenesis and growth as well as apoptosis of neoplastic cells, the search for compounds that inhibit the level/activity of Trx and/or TrxR and thus modulate the course of the neoplastic process is ongoing. It has been shown that many naturally occurring polyphenolic compounds inactivate elements of the thioredoxin system. In addition, the effectiveness of Trx is inhibited by imidazole derivatives, while the activity of TrxR is reduced by transition metal ions complexes, dinitrohalobenzene derivatives, Michael acceptors, nitrosourea and ebselen. In addition, research is ongoing to identify new selective Trx/TrxR inhibitors.

There are two different isoforms of Trx – a cytosolic isoform (Trx1) and a mitochondrial isoform (Trx2), which are found in different tissues of the human body. Trx1 is a 12 kDa protein that is biosynthesized as a 105-amino acid propeptide, which through an activation process, undergoes removal of an N-terminal methionine, leading to the generation of the 104-amino acid active protein that has valine at its N-terminus. The mitochondrial Trx isoform is biosynthesized as an 18 kDa protein containing 166 amino acids with a 60-amino acid sequence at its N-terminal that is responsible for directing the newly synthesised Trx molecule into the mitochondrion. After reaching the mitochondrion, the protein is hydrolysed with a 60-amino acid peptide cleavage, resulting in the 12.2 kDa active Trx2 protein.

Given the metabolic significance of Trx, the most important element of the molecule is its active centre (-Cys32-Gly-Pro-Cys35-) as it is responsible for the antioxidant function of the protein. The cysteines located in the active centre are arranged in such a way that Cys32 is more exposed to the outside while Cys35 is hidden in the core. This spatial distribution is important for the reducing action of Cys32, which leads to a disulphide bond between Trx and the protein to be reduced. Cys35 is responsible for the separation of the reduced protein molecule and the creation of a disulphide bond within the active Trx centre (Figure 2). The conservation of this sequence in the active centre is essential for the proper functioning of Trx and any amino acid change in the sequence (e.g. substitution of proline with serine or threonine) leads to a change in protein conformation, resulting in decreased stability of the molecule, which in turn, significantly affects the reduction potential of Trx. Apart from the cysteine residues located in the active centre of Trx, there are three additional cysteines in the structure of the protein at positions 62, 69, and 73. Therefore, in a situation of Trx deficiency and/or under oxidative conditions, it is possible to use...
other thiol groups for Trx reduction activities through the formation of a second disulphide bond between Cys62 and Cys69. Further oxidation on the other hand, would lead to the formation of a homodimer with a disulphide bond between the cysteines at position 73 of two Trx molecules

The enzyme responsible for reducing the oxidised form of Trx is thioredoxin reductase (TrxR). The reducing enzyme contains a FAD prosthetic group, NADPH binding domain, interface domain, and an active centre located in the C-terminus. The TrxR active centre contains one cysteine and one selenocysteine (-Gly-Cys497-Sec498-Gly-) and the FAD domain contains the sequence -Cys59-Val-Asn-Val-Gly-Cys64-, which enables electron transfer during a redox reaction. An important element for TrxR activity is the Sec498 located at the enzyme’s C-terminus. It has been shown that recombinant mutant TrxR containing cysteine instead of selenocysteine in the active centre showed a 100 times lower catalytic constant value in comparison to the wild type Sec-containing enzyme. It was also observed that the substitution of redox inactive serine in place of selenocysteine completely inactivates the enzyme. TrxR biological activity. TrxR molecules are arranged in space such that the C-terminal active site of one molecule is adjacent to cysteines present in the redox-active sequence at the N-terminal element of another molecule (Figure 3) – this is crucial for the function of the enzyme because such a spatial organisation allows for the free flow of electrons during the redox reaction. After recruitment of NADPH via the NAPDH binding domain of the enzyme, electrons are transferred to the FAD domain at the N-terminal end of the protein. The active redox sequence of one subunit of the enzyme transfers electrons to the active site located at the interface domain of the other subunit.

In humans, TrxR occurs in three isoforms: the TrxR1 isozyme which is present in the cytosol (55 kDa; 499 amino acids), the TrxR2 mitochondrial isozyme (56.2 kDa; 521 amino acids), and TrxR3 (65 kDa; 560 amino acids) which localises to the nucleus. TrxR2 contains a domain composed of 33 amino acids situated at the N-terminal portion of the enzyme, which is responsible for mitochondrial translocation of enzyme molecules. TrxR3 can reduce both Trx and glutathione (GSH) and is therefore referred to as thioredoxin glutathione – reductase (TGR). It contains a TGR structure at the N-terminal motif which contains an additional glutaredoxin domain so that the enzyme can act as a thioredoxin reductase, glutathione reductase, and glutaredoxin, the purpose of which is to reduce mixed disulphides in proteins produced by glutathionylation, among other processes.

The effective action of the Trx-dependent system is also linked to the presence of thioredoxin peroxidase (TPx), which in cooperation with Trx, can reduce lipid peroxides and hydrogen peroxide. TPx belongs to a family of proteins called peroxiredoxins (Prx), which contain in their active centre, a cysteine (Prx-SH) that is prone to oxidation. The peroxiredoxin molecule is converted to its oxidised form (Prx-SOH) as a result of peroxide reduction, which in reaction with another reduced peroxiredoxin molecule, forms a disulphide bond (Prx-S-S-Prx) with simultaneous release of a water molecule. TPx contains the Cys47 and Cys170 residues, which participate in peroxide reduction. The large distance between the cysteines makes it impossible for the formation of an intramolecular disulphide bond and as a result, the oxidised form of TPx, similar to TrxR, occurs in the form of dimers in which two intermolecular bonds in the Cys47-Cys170 system are formed. The effective action of TPx requires the presence of reduced Trx. The reduced form of Trx reduces TPx, which can then reduce peroxide molecules in the cell, protecting other molecules from the oxidative effects of ROS.

Although antioxidant activity is the main function of the thioredoxin-dependent system, the system also participates in cofactor activities and regulates the expression of many genes. Trx supports the transcriptional activity of the nuclear factor NF-kB – which can stimulate the growth of cancer cells – by promoting its interaction with DNA through reduction of the disulphide bond in one of the NF-kB subunits. Trx also binds to Apoptosis signal-regulating kinase (ASK1) – which regulates apoptosis under oxidative stress conditions – to cause its degradation. However, in the presence of ROS or tumour necrosis factor alpha (TNF-α), Trx and ASK1 are released. ASK1 stimulates cellular growth through the activation of kinases such as p38 and JNK, which stimulate the activity of the proapoptotic protein Bax and simultaneously inhibit the anti-apoptotic factor Bcl-2. This promotes the activation of caspase 3, which triggers apoptosis. Moreover, Trx, through the Reduction-oxidation factor 1 (Ref-1), modulates the activity of the transcription factor activator protein 1 (AP-1) to regulate the expression of genes involved in cell growth in response to...
Modification of the Trx-TrxR System

Natural physicochemical factors modulating Trx – TrxR system activity

The biological activity of the Trx-TrxR system depends primarily on endogenous blocking inhibitors that bind at least one of the system’s components. An example of such a Trx-binding molecule is the Thioredoxin Interacting Protein (TXNIP, also called thioredoxin binding protein-2, TBP-2 or vitamin D3 stimulated protein 1, VDUP-1), which binds to Trx. TXNIP has 391 amino acids, a molecular weight of 46 kDa, and contains two cysteines (at positions 63 and 247), which allow it to react directly with Trx. This interaction is key in regulating the Trx-dependent system and in regulating cellular homeostasis.

The formation of a disulphide bond between Cys247 in TXNIP and Cys32 in Trx leads to the formation of a stable complex and blockade of the Trx catalytic centre, resulting in inhibition of the antioxidant action of the redox protein. However, a TXNIP mutation that results in substitution of cysteine with serine at position 247 results in the elimination of its inhibitory effect on Trx. It is also important that TXNIP expression in acute myelogenous leukemia (AML) cells isolated from patients is decreased by up to 11 times and levels are also reduced in primary breast and colon cancers. For example, it has been shown that TXNIP expression in acute myelogenous leukemia (AML) cells isolated from patients is decreased by up to 11 times and levels are also reduced in primary breast and colon cancers.

Therefore, to prevent and pharmacologically treat tumours, compounds that inhibit Trx-dependent system activity are being sought, and mainly those with natural origins.

It has been found that the enzymatic activity of TrxR can be inhibited by some natural polyphenolic compounds including curcumin. Curcumin causes covalent modification of the C-terminal TrxR fragment, namely alkylation of both cysteine and selenocysteine. This irreversibly inhibits the activity of the enzyme, causing TrxR to lose its ability to reduce proteins through Trx, which promotes an increase in oxidative stress in cells. Similar effects have been observed with quercetin and myricetin, which like curcumin, can cause irreversible inhibition of TrxR activity. For myricetin, it has been observed that a TrxR mutant containing cysteine in position 498 does not prevent the oxidation of this

Figure 3. Scheme of arrangement of thioredoxin reductase homodimer.
flavanol. This suggests that the selenocysteine in the active centre of TrxR is crucial for the interaction between TrxR and flavanols\(^4\). Curcumin-induced alkylation of TrxR can have effects analogous to NADPH oxidase that involve significant increases in ROS production and increased oxidative stress\(^4\).

The only physical factor that has been found to inactivate TrxR is ultraviolet radiation. It has been shown that both UVA and UVB radiation from sunlight, even at a dose below the minimum erythema dose, causes inactivation of TrxR, which in turn, significantly reduces the antioxidant effect of the TrxR system\(^4\).

Among naturally occurring chemical compounds, some support the Trx-dependent system, leading to increased expression of its components. Among these is cannabidiol (CBD) – a compound found in the extracts of the Cannabis sativa L. plant – which has no psychoactive effect but has antioxidant, anti-inflammatory, and anticancer activity\(^4\). In vitro studies have shown that at a concentration of 4 \(\mu\)M, CBD leads to an increase in both Trx and TrxR expression, while reducing the expression of GSH-dependent antioxidant system components including GSH itself, as well as GSH reductase and peroxidase, suggesting that CBD action may promote the activity of the Trx-TrxR system\(^4\).

**Synthetic compounds that inhibit the activity of the Trx – TrxR system**

In addition to naturally occurring Trx-dependent system inhibitors, many synthetic compounds can also modify the functioning of the Trx-TrxR system by inhibiting the biological activity of Trx or its reductase. This includes compounds from the group of disulphide imidazole derivatives, naphthoquinone derivatives and Michael acceptors as well as several organic and inorganic complex compounds containing transition metal ions as the central atom (Figure 4) (Table 1)\(^3\).

**Synthetic inhibitors of Trx and TrxR**

Spiroketal naphthoquinone derivatives such as pleurotin can be used to inhibit the Trx-TrxR system (Table 1)\(^4\). Some compounds from this group can preferentially act as Trx inhibitors while others more effectively inactivate TrxR. Moreover, some of the compounds in this group are effective inhibitors of the Trx-TrxR system although they do not interact directly with either Trx or TrxR. This suggests that in addition to inhibiting the biological...
activity of Trx or TrxR, spiroketal derivatives of naphthoquinone may affect other cellular metabolic processes, e.g. by reducing HIF-1 expression levels51–53.

Synthetic inhibitors of Trx

Compounds from the group of alkyl-2-imidazolyl disulphides, including 1-methylpropyl-2-imidazolyl disulphide (known as PX-12 in formulation), which has been tested in clinical trials as a Trx1 inhibitor, can modulate the functioning of the Trx-TrxR system54,55. PX-12 has been tested in the treatment of advanced, therapy-resistant cancers (e.g. non-small cell lung cancer, pancreatic, breast, and gastrointestinal cancers, as well as multiple myeloma)56–57. The action of this group of chemicals is based on the thiol alkylation reaction of Cys32 and Cys35 of the catalytic centre and Cys73 outside the catalytic centre of Trx that they induce. It has been observed that the PX-12-mediated thiol alkylation reactions of Trx within Cys32 and Cys35 are reversible and rapid whereas reaction with Cys73 is irreversible and much slower58. This is important for the action of the Trx-TrxR system because the reaction with the cysteine residue located beyond the Trx catalytic centre causes the protein to be permanently inactivated. This results in a decrease in the expression of factors whose activity is associated with Trx antioxidant activity directly (e.g. factors induced by HIF-1α under hypoxia) as well as indirectly (e.g. vascular endothelial growth factor, VEGF, which is induced by HIF-1α)59,60. Additionally, inhibition of Trx and impairment of its binding to ASK-1 leads to the activation of mitogen-activated protein kinase (MAPK)60. Together, these effects lead to a significant induction of apoptosis. In the case of simultaneous administration of PX-12 and cis-diaminodichloroplatin (a TrxR inhibitor), increased inhibition of tumour growth was observed in comparison to administration of platinum complex only56,59,60 (Figure 5).

Synthetic inhibitors of TrxR

A significant group of compounds that can inhibit the activity of TrxR include complexes containing metal ions, mainly transient ones. Among chemicals that inhibit TrxR activity, some of the most effective are platinum and gold complexes62,63. Platinum complexes (cis-platinum, carboplatin) – which are used in the treatment of many cancers – irreversibly inhibit the activity of TrxR while maintaining the activity of other reductases (e.g. glutaredoxin or GSH reductase). This is because platinum complexes show a greater affinity for the selenol group (present in the TrxR active centre) compared to the thiol group (located in the GSH reductase active centre)64. Gold compounds also inhibit TrxR activity, likely based on a similar mechanism as platinum complexes65. In vitro studies have shown that auranofin – a complex containing gold at +1 oxidation level – is capable of inhibiting TrxR at a low, nanomolar level concentration whereas such an effect on glutathione reductase (GR) was only observed at higher micromolar concentrations66. Differences in auranofin concentrations needed to inhibit these enzymes were attributed to the presence of selenocysteine in the C-terminal motif of TrxR and its absence in GR. Additionally, it has been observed that during auranofin application, hydrogen peroxide production and cytochrome C secretion increases, which results in a change in the redox environment and consequently, apoptosis67,68. It has been suggested that gold ions that bind to cysteine and selenocysteine in the C-terminal motif of TrxR create a -Se-Au-S- motif, blocking the possibility of the enzyme to form disulphide bond69.

In addition, auranofin – apart from increasing the generation of hydrogen peroxide – can also lead to an increase in intracellular concentrations of calcium ions. Since an increase in ROS promotes the transport of calcium ions across the cell membrane65, it is suggested that hydrogen peroxide may cause the release of intracellular calcium ions, which are involved in regulating metabolic functions in cells70. Calcium ions regulate the expression of various cytokines including IL-2 and IL-8, as well as COX2 and prostaglandin E2, influencing inflammatory processes in cells. Calcium gradients also participate in the homeostasis of permeability barriers71,72. Based on circular dichroism spectra, it was found that calcium ions acting on the TrxR1 isoform cause a change in the conformation of the enzyme, which leads to a reduction in its cytosolic activity, whereas such changes were not observed for TrxR2. Moreover, it is suggested that the oxidised form of Trx, acting by way of feedback, may partially protect TrxR against inactivating calcium ions73.

Lanthanum (III) chloride (LaCl3) also shows an inhibitory effect on TrxR activity, similar to that of calcium ions, which can be explained by the similarities between Ca2+ and La3+ ions. Like calcium ions, lanthanum ions have a stronger affinity for the cytosolic TrxR isoform than the mitochondrial reductase, which manifests itself in lower required concentrations to reduce TrxR activity, and increasing ROS levels only affect relatively low concentrations of lanthanum ions74,75. Higher concentrations of La3+ ions (similar to calcium ions) inhibit TrxR, whereas no increase in ROS levels are observed, which suggests that lanthanum compounds may also act as scavengers of ROS. It is important to note that LaCl3 does not react directly with cysteine or selenocysteine in TrxR but forms a link with the enzyme outside its active centre, which changes the spatial arrangement of the polypeptide TrxR chain. This is explained by the fact that according to the theory of hard and soft acids and bases (HSAB), the La3+ ion is a hard acid that reacts with groups classified as hard bases (e.g. carboxylate ions or hydroxyl groups)76. Additionally, it has been observed that LaCl3 does not show an inhibitory effect on GR activity, even when significant concentrations of the salt are used75.

Other compounds that inhibit TrxR activity include organic complexes containing tin (Sn) or ruthenium (Ru) ions at +4 and +3, and +2 oxidation levels, respectively76,77. Like other metal ions, ruthenium compounds inhibit the activity of only the cytosolic isoform of the TrxR, while the mitochondrial isoform does not show significant changes in activity even after administration of high concentrations of ruthenium76. However, some Ru (II) compounds show an antiproliferative effect on melanoma cells similar to the effects of cisplatin, whereas other ruthenium compounds have more significant anti-proliferative effects than cisplatin. In the case of multi-drug resistant lung cancer cells insensitive to cisplatin, ruthenium complexes have been shown to significantly inhibit TrxR activity. Also, these compounds were shown to...
inhibit the activity of TrxR more effectively than auranofin\textsuperscript{78}. Tin compounds also inhibit the activity of TrxR; however, this activity is much weaker compared to gold or platinum compounds\textsuperscript{77}.

The activity of TrxR is also inhibited by motexafin gadolinium (MGd), which is a drug used in anticancer therapy. Direct binding of MGd to TrxR has been observed, but not in the active centre of the enzyme\textsuperscript{79}. It has also been found that in the presence of suitable proteins (e.g. some metallothioneins), the administration of MGd can lead to the release of zinc as a byproduct of redox reactions, which is also an inhibitor TrxR. Thus, in addition to its direct inhibitory effects, MGd can also inhibit TrxR indirectly through generation of zinc ions\textsuperscript{79,80}.

In addition to the abovementioned organometallic compounds, TrxR activity is also inhibited by compounds containing rhodium (Rh) and copper (Cu) ions. It has been shown that compound complexes containing Rh(I) in their structure that inhibit the activity of TrxR also have an antiproliferative effect on cancer cells such as breast adenoma and colorectal cancer cells\textsuperscript{81,82}. However, copper (I) alkyl phosphine complexes have greater cytotoxicity towards cancer cells than analogous compounds containing Au\textsuperscript{+} and Ag\textsuperscript{+} as a central ion, and higher cytotoxicity than the reference compound cisplatin\textsuperscript{83}. The fact that Cu(I) complexes inhibit TrxR activity to a much lesser extent than the reference compound suggests that the toxic action of Cu-containing complexes against cancer cells does not result from apoptosis, but is caused by impairments to systems that regulate redox homeostasis.

Ebselen – an organic compound capable of reducing lipid peroxides and H\textsubscript{2}O\textsubscript{2} – is another type of Trx inhibitor that results in the production of selenolic acid (EbSeOH). In mammalian cells, this compound is a substrate for TrxR, which reduces EbSeOH to an active selenolic form (EbSeH). Ebselen is a selective inhibitor that exclusively inhibits the activity of the bacterial form of TrxR, leading to an increase in ROS production, ultimately resulting in

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image.png}
\caption{Connection of the thioredoxin-dependent system to the cascade of transformations leading to cell apoptosis [according to Matsuzawa\textsuperscript{61} in own modification].}
\end{figure}
the death of bacterial cells. This is important for the potential use of ebselen for the treatment of bacterial infections, but the compound also has a protective effect on mammalian cells.

The ability to interact and modify thiol and selenium TrxR groups is exhibited by electrophilic organic compounds from the groups of nitrosoareas, dinitrohalobenzenes, and polyphenols. These compounds may also bind to other functional groups, simultaneously inhibiting the action of other pro-survival enzymes in the presence of electron withdrawing groups in their structures makes them act as Michael acceptors and thus have the ability to inhibit the activity of TrxR. Therefore, the search for other TrxR inhibitors that will inhibit both thioredoxin reductase and carbonic anhydrase is ongoing. Such a group of compounds includes the sulfocoumarins and other Michael acceptors which are classified as Michael acceptors and thus have the ability to inhibit the activity of TrxR.

Another pharmacologically important group of TrxR inhibitors are compounds that are classified as Michael acceptors. These include compounds containing electron-withdrawing groups (for instance ester or carbonyl group) connected with alicenes which interact directly with the active centre of the TrxR. The compound signed as DVD-445 deserves special attention because of its preferential effect on easily proliferating cancer cells. This effect was noticed during a cell viability assay performed on human keratinocytes (HaCaT) as well as neuroblastoma (SH-SY5Y) and glioblastoma (U87 and U87-TxR) cell lines. Despite the effective selective action of DVD-445 against cancer cells, it has been observed that neuroblastoma cells remain viable at approximately 80%. In addition, some DVD-445 analogues also show inhibitory activity against TrxR activity, but not in cancer cells. Therefore, the search for other TrxR inhibitors that will simultaneously inhibit the action of other pro-survival enzymes in relation to cancer cells is ongoing. Such a group of compounds includes the sulfocoumarins and other Michael acceptors which inhibit both thioredoxin reductase and carbonic anhydrase. The presence of electron withdrawing groups in their structures makes them act as Michael acceptors and thus have the ability to inhibit the activity of TrxR. A similar effect was observed for a synthetic compound that contains both the Michael acceptor group (responsible for the inhibition of TrxR) and the motif responsible for inhibiting carbonic anhydrase, which is very important in the process of carcinogenesis, because carbonic anhydrase protects cancer cells against acidification and hypoxia, preventing them death. This "hybrid" exhibits significant cytotoxicity to the pancreatic cancer cell line (PANC-1) compared to co-administered combinations of TrxR inhibitors and carbonic anhydrase inhibitors. Consequently, due to the efficacy and preference for cancer cells, the synthesis and further research on Michael acceptors indicate the possible emergence of a compound effective in the treatment of highly multi-drug resistant cancers.

### Table 2. Summary of compounds inhibiting the efficiency of thioredoxin reductase.

| Metal ion | Type of compound/complex | TrxR1 | TrxR2 | Results of the action | Type of tested cells | References |
|-----------|--------------------------|-------|-------|-----------------------|---------------------|------------|
| Pt<sup>2+</sup> | cis-platin carboplatin oksaliplatin | +     | +     | proliferation decrease | breast cancer, ovarian cancer, lung cancer, leukaemia, mice sarcoma | 42,83      |
| Au<sup>3+</sup> | auranofin and its derivatives | +     | +     | increase in ROS level | ovarian cancer, cervical cancer, lung cancer | 62,65,66,67,69,95 |
| Ru<sup>3+</sup> | thiazole and polypyridyl complexes | ++    | +     | proliferation decrease, apoptosis, change of redox environment | breast cancer, lung cancer, liver cancer, melanoma | 76,78,96 |
| Sn<sup>4+</sup> | motexafin gadolinium (MGd) | +     | +     | release of Zn<sup>2+</sup> from metallothioneins, increase in ROS level, apoptosis | lung cancer, ovarian cancer, B-cell lymphoma | 79,80 |
| Rh<sup>3+</sup> | carbene rhodium derivatives | +     | +     | proliferation decrease, increase in ROS level | neuroblastoma, colorectal cancer, liver cancer, pancreatic cancer, breast cancer, prostate cancer, colorectal cancer | 81,82 |
| Cu<sup>+</sup> | | +     | +     | parapoptosis | breast cancer, ovarian cancer, cervical cancer, lung cancer, colorectal cancer, melanoma | 83 |
| Se<sup>4+</sup> | ebselen (EbSeOH) | +     | +     | apoptosis | bacteria of the families H. pylori, M. tuberculosis, S. aureus, B. subtilis | 84 |
| La<sup>3+</sup> | lanthanum chloride | ++    | +     | increase in ROS level, redox balance control | ovarian cancer | 75 |

The "++" symbol indicates an enzyme isoform for which a more effective inhibition of the enzyme activity has been observed.
Conclusion

The Trx-TrxR system is essential to maintaining cellular redox homeostasis. Cellular metabolism in normal physiological, as well as pathophysiological, conditions including carcinogenesis, depends on the efficiency of this system. Since the Trx-dependent system can both contribute to the intensification of angiogenesis, tumour growth, resistance to therapy, as well as drive changes that result in the death of cancer cells by apoptosis, the search for compounds that are inhibitors of Trx and/or TrxRs could modulate the tumoral activity of the Trx-dependent system. The functions of the Trx- and GSH-dependent systems complement each other, so a decrease in the performance of a Trx-dependent system usually increases the performance of a GSH-dependent system. Therefore, ideal inhibitors would simultaneously limit the function of both antioxidant systems.

Disclosure statement

No potential competing interest was reported by the author(s).

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