The value of proteasome inhibition in cancer

Can the old drug, disulfiram, have a bright new future as a novel proteasome inhibitor?

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The major approach to the development of anticancer drugs involves searching for new compounds, efficient against malignancies, which are not, as yet, used clinically. This strategy is time-consuming and expensive. Recent studies have disclosed a surprising, but mechanistically consistent, anticancer activity of disulfiram (antabuse), a drug used for about 50 years in the treatment of alcoholism. Disulfiram has been successfully used to suppress hepatic metastases originating from ocular melanoma. The pharmacokinetics of disulfiram and its pharmacological profile in cancer cell lines and in cancer cells obtained from patients is well known. Disulfiram is a readily available and inexpensive substance whose adverse effects are negligible, compared to classical cancerostatics. In addition, the inhibitory potency of disulfiram against the proteasome conforms to current anticancer strategies and represents a new, promising approach to proteasome inhibition.

The proteasome, an Achilles' heel of cancer?

The proteasome is one of the most fascinating and important topics currently addressed in either cellular or pharmacological sciences. There are hundreds of articles pertaining to proteasome structure, its roles in cellular signaling, in diseases and so on. It is quite probable that published research in this field deserves Nobel Prize distinction. Incidentally, the 2004 Nobel Prize in Chemistry was awarded to Aaron Ciechanover, Avram Hershko and Irwin Rose 'for the discovery of ubiquitin-mediated protein degradation'. This degradation is mediated by various biologically distinct parts of the proteasome. Such a process is an important event in the cell as Avram Hershko highlighted in his Nobel Lecture [1]: 'By the late 1960s, it became apparent that normal proteins are also degraded in a highly selective fashion. The half-life times of different proteins ranged from several minutes to many days, and rapidly degraded proteins usually had important regulatory functions.' The ubiquitin–proteasome system (UPS) is a complex and dynamic intracellular protease, responsible for the degradation of about 90% of proteins in the cell and plays a crucial role in maintaining normal cellular homeostasis. Despite this lack of specificity, the UPS (especially the proteasome) has emerged as an attractive pharmacological target for the development of novel anticancer drugs [2,3]. We still do not know, however, what, ultimately, the long-term success of proteasome inhibition will be in cancer therapy. This commentary will not address this question, nor will it be aimed at a comprehensive summary of current UPS research. Instead, we would like the readers to focus on the old drug disulfiram (antabuse), which has been used for about 50 years in the treatment of chronic alcoholism [4] and its potential therapeutic application as a proteasome inhibitor in cancer therapy. Disulfiram is safe [5], cheap and appears to function by a distinct biological mechanism from that of established proteasome inhibitors. Moreover, it has been successfully used

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(with zinc gluconate) in a human patient with metastatic melanoma [6].

At this point, it is necessary, at first, to sketch a picture of the UPS and, in particular, the proteasome. Insofar as there are many comprehensive reviews on this topic, we portray (Fig. 1) the 'basic dogma' of UPS. For a more in-depth discussion on the UPS, see [7].

Degradation of proteins through the UPS

The proteins that are to be degraded or processed must undergo labeling with ubiquitin at Lys48. There are, however, at least two known exceptions:

1. Oxidized proteins can be degraded without ubiquitin conjugation [8] and
2. FAT10-labeled proteins are intended for proteasome degradation in a nonubiquitin-mediated fashion as well [9]. Moreover, proteins can be degraded through an alternative, PA28γ (the γ-subunit of 28-kDa proteasome activator or 11S cap) proteasome pathway, independently of ubiquitin conjugation [10,11].

The labeling machinery is composed of three distinct types of enzymes. First of them (denoted E1 and called 'ubiquitin-activating') binds a molecule of ubiquitin in an ATP-dependent manner and transfers it to the 'ubiquitin-conjugating' enzyme E2. Such a complex (E2 + ubiquitin) translocates near to a site of a protein to be degraded where 'ubiquitin ligase' E3 is bound. The ubiquitin molecule is transferred by this enzyme to the substrate and every other such molecule is bound to the previous one, forming a linear chain. There are many E3s that can be categorized, according to their modular structure, into four groups: RING-finger (really interesting new gene); HECT (homologous to E6AP carboxyl terminus); U-box and PHD-finger (plant homeodomain).

If the ubiquitin chain contains at least four ubiquitins, it is recognized by the proteasome and recruited for its 19S (Fig. 1) regulatory particle (RP). This process requires so-called 'multiubiquitin chain binding proteins' with a surprising degree of substrate specificity [12]. The recruited protein must lose the ubiquitin chain to enter the active (core) particle (CP) of the proteasome, so there are two deubiquitinating enzymes within RP, Ubp6 (ubiquitin-specific protease-6) and Poh1 (pad one homolog-1), which is known as RP number 11, or Rpn11, in yeast. Ubp6 can both cleave the ubiquitin chain from the substrate and delay its degradation [13]; moreover, it is involved in a chain remodeling, which seems to be another important level of proteasome selfregulation [14]. Ubp6 is not, however, an integral subunit of RP, so Poh1

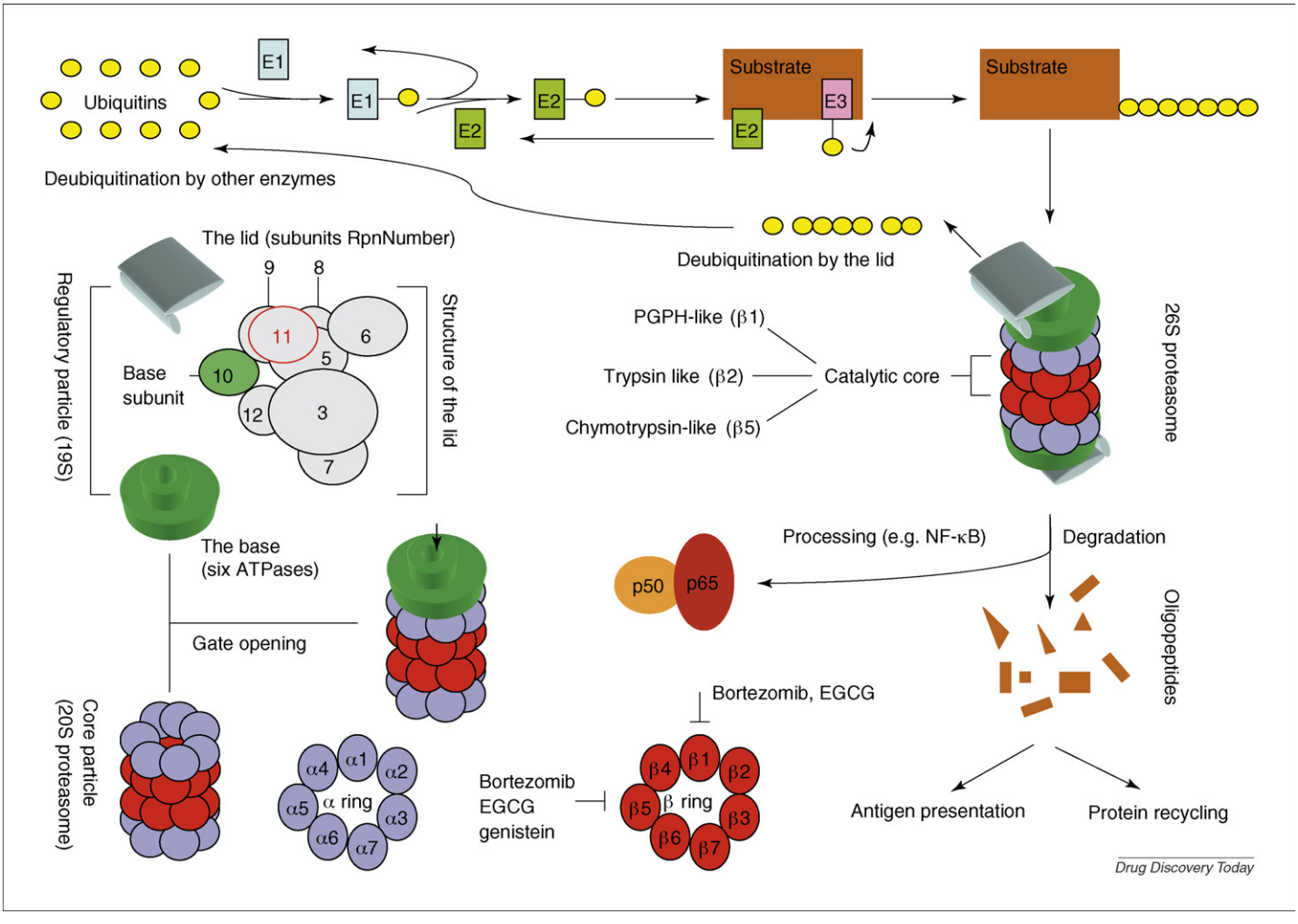

FIGURE 1

The ubiquitin–proteasome system and the structure of the 26S proteasome. Rpn11/Poh1 (in red letters) is possibly the target for dithiocarbamate ligands/complexes. NF-κB: nuclear factor-κB; PGPH: peptidylglutamyl peptide hydrolyzing; EGCG: epigallocatechin-3-gallate.

seems to be more important for the intrinsic deubiquitination activity of the proteasome lid. The so-called lid is one of the two RP parts; the second is simply named the base and serves to open the CP gate [15]. Thus, if protein is recruited, deubiquitinated and unfolded, it can enter CP where is lysed into oligopeptides, which can be transported to the endoplasmic reticulum, bound to MHC (major histocompatibility complex) class I molecules and subsequently presented on the cell surface as antigens [16]. More commonly, the generated proteolytic fragments are used for new protein synthesis [17]. The ubiquitinated protein, however, is not required to be fully degraded; it can be partly processed by the proteasome to (for example) promote the protein's role in cell signaling, such as is the case for NF-κB (nuclear factor-κB).

The proteasome and proteasome subunits are involved in various cellular processes, such as aging [18], HIV-transcription regulation [19], or transcriptional activation [20] and circadian rhythm control [21]. Hence, it is quite incredulous that inhibition of such a multifunctional and vital zome[3] can be pursued as a novel strategy for the treatment of human malignancy; however, facts demonstrate that, not only is it possible, but also it is a very effective strategy.

The exciting success of bortezomib

Let us cite the Millenium Pharmaceuticals *Annual Report 2006* (pg 4)[4]: 'Last year, Millenium successfully expanded the VELCADE® label to provide a therapeutic option to an entirely new group of patients in need. In addition to maintaining its status as the U.S. market leader in relapsed multiple myeloma – with more than 50,000 patients worldwide treated to date – VELCADE® was approved for the treatment of previously treated mantle cell lymphoma, an aggressive, incurable form of NHL that affects 10,000 people in the U.S. alone. VELCADE® is the only therapy approved for this indication.' The remarkable story of bortezomib (VELCADE®) is well known (cf. NCI Success Story: Velcade). This drug, a 'first in class' inhibitor of CP, is a boronic acid dipeptide discovered in 1995. It was submitted to NCI's Developmental Therapeutics Program and shown to be potent against all cell lines tested. The reversible inhibition of proteasomal function (approximate inhibition of 70%) was reached in MM (multiple myeloma) patients with optimal anticancer efficacy. This subsequently led to its approval in May 2003 [22]. Clinical research is ongoing to establish bortezomib's ability to treat other cancers, either as monotherapy or in combination with regimens [23].

Three β-rings (Fig. 1) are responsible for the peptidase activity of CP: chymotrypsin-like (β5 subunit cleaves after hydrophobic side chains), peptidylglutamyl peptide hydrolyzing (PGPH)-like (β1 subunit cleaves after acidic side chains) and trypsin-like (β2 subunit cleaves after basic side chains). There are many agents known to inhibit CP activity with promising therapeutic effects against various cancers: for example, genistein (in clinical trial at Barbara Ann Karmanos Cancer Institute), epigallocatechin-3-gallate (EGCG) (in clinical trial at Jonsson Comprehensive Cancer Center), metal compounds, salinosporamide A (a β-lactone) and the irreversible inhibitor PR-171 (an epoxomicin derivate) [24–29]. From crystallographic studies, the molecular basis of CP inhibition by bortezomib and other compounds is well known [30]. As authors of [30] suggest, future research should be focused more on 19S inhibitors: 'Though nowadays there exist quite a number of selective and efficient proteasomal inhibitors, the toxic side effects of these compounds strongly limit their potential in possible disease treatment. One possibility to influence proteasomal substrate specificity might be the modulation of activity of the 19S regulatory particle and associated proteins.' In this review, we will try to show (fourth section) that disulfiram could inhibit Poh1.

Cancer cells are more sensitive to proteasome inhibition than normal cells. There are many papers proposing the most probable cellular pathways responsible for this selectivity that appears to act independent of the proliferative status of the cell [31,32]. The proteasome plays various key functions in cellular signaling and, as our knowledge of cellular networks expands, it may take on increasingly complicated roles. It is clear, however, there must be some feature(s) of cancer cells, lacking in normal cells, that sensitizes them to proteasome inhibition. NF-κB, for instance, is often suggested to be a reason for such disparity in cell responsiveness because of its role in tumor promotion, survival and metastasis. Moreover, this factor, which can be activated by anticancer drugs, offers a good explanation for the known ability of proteasome inhibitors to sensitize malignant cells to standard therapeutics [33,34]. Likewise, a role of NF-κB in angiogenesis can also make clear their antiangiogenic properties [35]. Therefore, we will focus on the NF-κB pathway in the light of current knowledge. We shall see (Fig. 2) new personages in still not well-known stories.

Is NF-κB the answer?

The discovery of the role of *Helicobacter pylori* in the pathogenesis of gastric ulcers (2005 Nobel Prize in Physiology or Medicine) has reopened the old question (cf. Virchow's widely known opinions) of whether there is a link between a chronic inflammation and tumorigenesis [36]. Without wishing to oversimplify this, it may well be that the answer to this question seems to be just NF-κB. Many reports have highlighted how the function of NF-κB in cancer and in response to therapy can vary.

In fact, NF-κB constitutive activation (because of genetic abnormalities) is characteristic of many malignant tumors, for example, MM [37,38]. The NF-κB pathway seems to be well known today [39] and described in many papers, especially the so-called canonical (or classical) role. This pathway (Fig. 2 'pro-cancer pathway') requires the proteasome to cleave inhibitor-κB (IκB) and, thereby, releases the heterodimer p50:p65 for nuclear translocation, leading to gene transcription. Such NF-κB activation promotes cancer development through the expression of cell cycle genes, apoptosis inhibitors, invasive proteases and so on [40]. Of course, the nuclear action of NF-κB, as well as the triggering of the canonical pathway and its cytoplasmic consequences, is regulated in a very sophisticated manner. For instance, a new switch between NF-κB and p53 has been recently described [41].

Hence, if NF-κB requires proteasome activity, proteasome inhibition leads to the blockage of NF-κB and cancer cell death. There are, however, new, proteasome-independent p65 pathways (Fig. 2). One of them is activated through MEKK3 (MAP/ERK kinase

[3] cf. 'Zomes IV – The 4th International Symposium on the COP9 Signalosome, Proteasome and eIF3 – at the Interface between Signaling and Proteolysis' held on the Yale University, 18–21 June 2006.

[4] http://www.library.corporate-ir.net/library/80/801/80159/items/249644/2007_MLNM_10K.pdf.

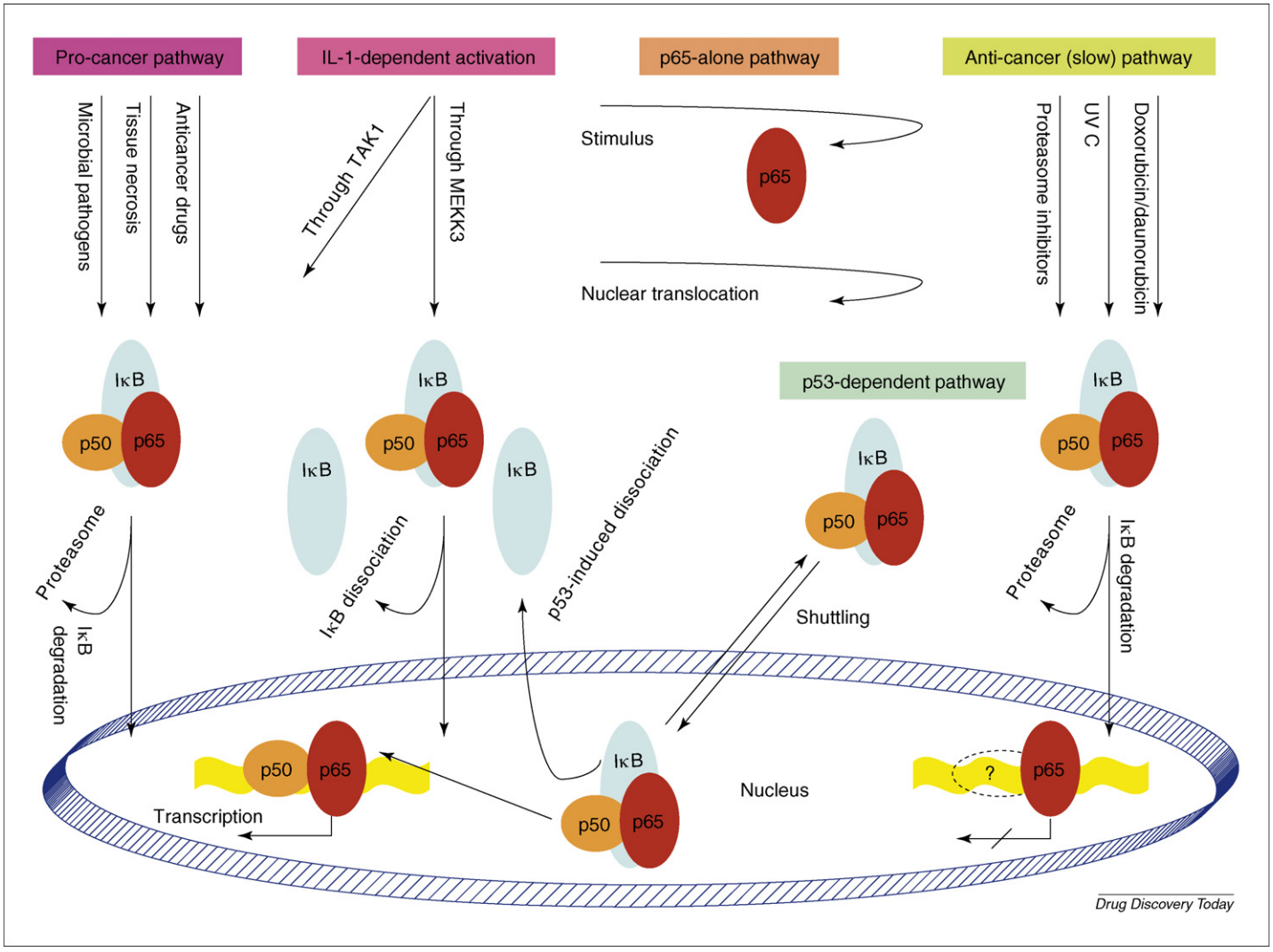

FIGURE 2

Proteasome-dependent and -independent p65 NF-κB pathways (as viewed in 2007). IκB: inhibitor-κB; TAK1: transforming growth factor activated kinase 1; MEKK3: mitogen-activated protein/ERK kinase kinase 3; IL-1: interleukin 1.

kinase 3) and does not require IκB degradation [42,43]. This mode of p50:p65 activation seems to be involved in innate immunity against cancer [44]. Additionally, a p65-alone pathway has been recently described: p65 is phosphorylated at serine 536 and thereby will not associate with IκB and p50; it can be translocated to the nucleus where it triggers gene expression [45]. Biological significance and details of this signaling remain unknown. More probably, the phosphorylation is not the only reason for p65 release from complexes with IκB and p50 because this phosphorylation is noted during the canonical pathway activation as well [46].

It is known that p50:p65:IκB complex can shuttle in and out of the nucleus where IκB can be ubiquitinated for cytosolic degradation by proteasome [47]. Recently published work shows that p65 within the nuclear p50:p65:IκB can be phosphorylated on serine 536 by p53-stimulated RSK1 (ribosomal S6 kinase 1). This leads to the release of p50:p65 from the complex (without IκB proteasomal degradation) and heterodimer action on cognate NF-κB enhancers [48]. The authors demonstrate that doxorubicin and etoposide activate just this NF-κB pathway and, as a result, also explain the proposed [49] anticancer activity of p53-mediated NF-κB activation. Moreover, this pathway is relatively slow (measurable in hours), in contrast to the canonical pathway (measurable in minutes), similar to another purported role for NF-κB 'anticancer' signaling.

This signaling, however, requires the intact proteasome and suppresses NF-κB-dependent transcription (Fig. 2) by, paradoxically, p65 NF-κB induction. Thus, ultraviolet light (UV-C) and daunorubicin induce the p65 association with histone deacetylases [50]. On the contrary, doxorubicin was shown to produce poorly phosphorylated and acetylated p65, which blocks NF-κB in a histone deacetylase-independent manner [51]. Recently, an article has illustrated a great heterogeneity in the NF-κB response to different drugs, even within a single tumor cell line [52].

Again, paradoxically, whereas it can be suggested that the proteasome-dependent pathway of p65 NF-κB activation should be inhibited by proteasome inhibitors, some studies have described the activation of such pathway (in hours) by bortezomib and three other proteasome inhibitors in endometrial carcinoma cell lines [53]. Which mechanism causes the treatment-induced

cell death is not clear. It is hard to say whether NF-κB really triggers transcription, because the authors tested it only on transfection level (not on cell's own DNA). Does activated p65 interfere with histone deacetylases, or do proteasome inhibitors attenuate NF-κB target gene expression at the level of mRNA? Interestingly, what can be stated is that the inhibitors trigger p65 phosphorylation on serine 536. Well, the role of this phosphorylation becomes more and more mysterious (cf. [54]).

The simple scheme of NF-κB inhibitors' antitumor activity, as we can see from current research, is no longer plausible (cf. [55]). The next phase will be to try to show that compounds widely used as NF-κB inhibitors might actually be proteasome inhibitors and that this can be the reason for their promising future(s) in clinical oncology.

Dithiocarbamates: standard inhibitors of what?

Not only disulfiram, but also its monomer, DDTC (diethyldithiocarbamate) (Fig. 3), is known in medicine (as ditiocarb or imuthiol [56]). Disulfiram, DDTC and their derivatives (dithiocarbamates) show rich coordination chemistry with main group and transition metals [57,58], which can explain most of their various biological activities [59].

Dithiocarbamates are widely used as inhibitors of canonical NF-κB pathway both *in vitro* and *in vivo* (many articles have been published, with the most important ones outlined in [59]) despite their lack of specificity. Since the beginning of this research in the early 1990s, it has been suggested that dithiocarbamates can block the release and degradation of IκB [60], possibly because IκB phosphorylation is abolished [61]. A popular explanation, that still persists, is that the dithiocarbamate effect on the NF-κB pathway is through oxidative stress attenuation (see [59] for details), but this birds-eye view has been increasingly replaced by novel molecular-level findings.

In the previously mentioned case of a patient with metastatic melanoma [6] the disulfiram effect was augmented by zinc ions. How can such a phenomenon be explained? Disulfiram is a zinc chelating agent [62] and dithiocarbamate complexes with Zn^{2+} are potent proteasome inhibitors [63] with antimelanoma activity [64]. Moreover, recent work has shown that copper (in line with [6]) complexes with dithiocarbamates inhibit proteasome activity and induce apoptosis in breast cancer cells (but not in normal breast cells) [65]. This complex also blocks proteasome function in prostate cancer cells [66].

According to another article, disulfiram complexed with Cu^{2+} selectively inhibits proteasome function in breast cancer cells (but not in immortalized human breast cells) and disulfiram alone induces, through targeting the proteasome, apoptotic cell death in human breast tumor xenografts [67]. This *in vivo* antitumor action of disulfiram was explained as follows: 'Cancer cells and tissues, which contain elevated copper and more dependent on proteasome activity for their survival, should be very sensitive to treatment with disulfiram and other copper binding compounds.' Although the authors show that disulfiram alone had no effect *in vitro* in their system, different results have been described for studies in HEK 293 ZsGreen cells [68]. Thus, with respect to the pleiotropic biological actions of disulfiram and dithiocarbamate [59], one might expect that a new hypothesis of proteasome targeting needs to be generated. The *in vitro* effect of disulfiram can, of course, depend upon experimental conditions, for example, how available are zinc or copper ions for complex formation in the medium or in the cell?

Is any protein sensitive to both metals (metals in aqueous milieu = coordination particles) and ligands in the proteasome? The canonical NF-κB pathway is (in a cell-free system) blocked by dithiocarbamates via E3 ubiquitin ligase inhibition [69] and this ligase depends (although some controversy exists with respect to this) on CSN5 (COP9 signalosome subunit 5) known also as Jab1 (jun activating binding protein 1) [70–73]. CSN5 and the key proteasome subunit Poh1/Rpn11 (for the arrangement of proteasome lid see [74] and Fig. 1) both have the JAMM domain [75], the active site sensitive to both metals (zinc acetate) and ligands (1,10-phenanthroline) [76,77]. The hypothesis that dithiocarbamate complexes can be inhibitors of JAMM domain proteins, formulated in [59], is being tested at Caltech (in the laboratory of Deshaies).

Taking all these into consideration, dithiocarbamates would seem to be primarily proteasome inhibitors and their ability to inhibit NF-κB inhibition is secondary. Moreover, synthetic complexes of Zn^{2+} with DDTC can trigger p65 nuclear translocation (in minutes) and cell death – for details and proposed explanation see [78]. Proteasome inhibitors, as we already know, do not have to act as NF-κB inhibitors, but can be even its activators. Thus, when

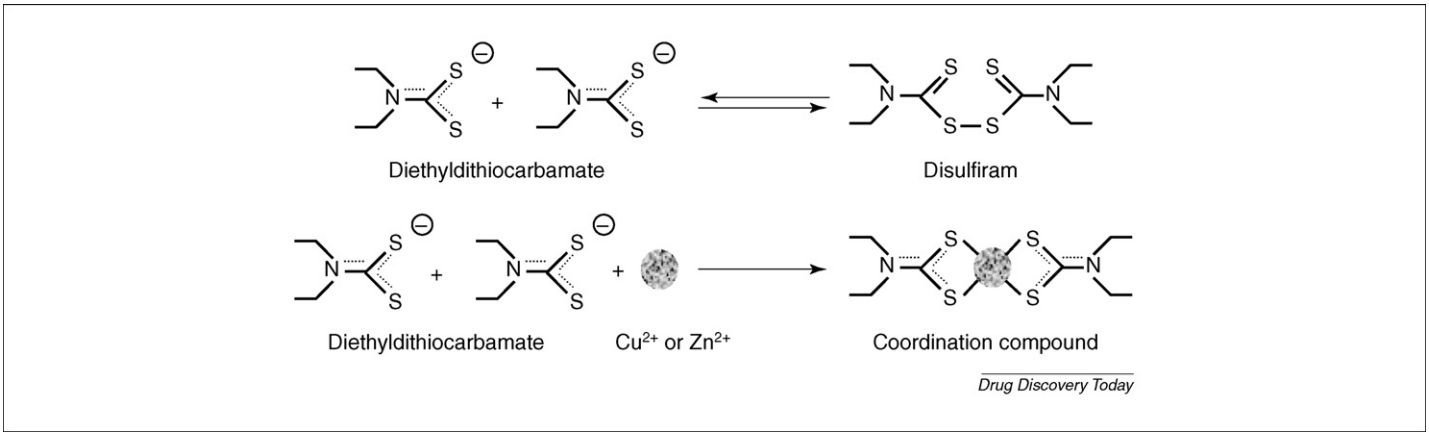

FIGURE 3

Dithiocarbamates can undergo oxidation, transforming themselves to thiuram disulfides (e.g. disulfiram) or react with metals to form coordination compounds.

using dithiocarbamates in cell experiments, it is important to establish whether they inhibit the proteasome and consider the many consequences of such inhibition.

New potency of an old drug

Anecdotally, a patient has been successfully treated for metastatic melanoma with disulfiram [6]; however, what would be the justification for large-scale clinical trials of disulfiram in the treatment of cancer patients?

In *Nature* in August 2007, Chong and Sullivan outline a major driver [79]: 'Because existing drugs have known pharmacokinetics and safety profiles and are often approved by regulatory agencies for human use, any newly identified use can be rapidly evaluated in phase II clinical trials, which typically last two years and cost $17 million.' Moreover, it seems to be better if an anticancer drug is able to suppress tumor development through multiple mechanisms. This is the case of disulfiram. It blocks drug resistance [80] as a single compound, which 'neutralizes the effect of both Pgp and MRP1 by three independent mechanisms at two different levels' [81]. Moreover, disulfiram as an NF-κB inhibitor enhances the effect of 5-fluorouracil in colorectal cancer cell lines (however, it does not inhibit IκB degradation) [82].

There are two ongoing clinical trials of disulfiram. The first one (based on targeting of glutathione metabolism) is being performed at Chao Family Comprehensive Cancer Center with patients suffering from multiple melanoma. The second one is continuing at Meir Medical Center with patients suffering from lung cancer and is motivated by the antiangiogenic properties of disulfiram [83]. In fact, disulfiram inhibits angiogenesis *in vivo* due to, it seems, direct interaction with matrix metalloproteinases through its ability to chelate zinc [62]. Might such a zinc complex (by analogy see [84]) inhibit proteasomes?

Given that disulfiram can inhibit JAMM domain proteins, it would introduce a new approach to proteasome inhibition, which seems to be of topical interest (according to scientists from Millenium Pharmaceuticals) [85]. Further, a poor prognosis expression pattern of 512 genes in breast cancer is induced by coordinate amplifications of MYC and CSN5 [86]; hence, CSN5 can be a useful target for cancer therapy (cf. [87]).

The pharmacological profile of disulfiram in cancer cell lines, and in cancer cells from patients, is currently known and encourages further clinical trials [88]. Moreover, it might be possible to identify alcoholics who suffered from cancer, who were treated with disulfiram whose tumors were suppressed. We hope that it may be possible to identify such cases.

Finally, the challenging reasons for clinical trials of disulfiram, both alone and with other drugs, arise even despite the fact that it may not be of commercial interest. Simply, the trials should be done in the interest of human beings with cancer.

Of course, there are also many incentive questions in proteasome and NF-κB research; and this is just what science needs on its never-ending way to a new (in Kuhn's words [89]) 'scientific revolution'.

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