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Research paper

Ridaifen-F conjugated with cell-penetrating peptides inhibits intracellular proteasome activities and induces drug-resistant cell death

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ABSTRACT

Ridaifen-F (RID-F) potently inhibits proteolytic activities of the 20S proteasome but poorly inhibits those of the 26S proteasome. Here, we report preparation of several conjugates in which various peptides are connected to RID-F. Conjugates with peptides consisting of seven amino acid residues significantly inhibited the 26S proteasome. Particularly, RID-F conjugated to an octaarginine peptide (R_8, a so-called cell-penetrating peptide) inhibited intracellular proteasome activities and induced cell death in drug-resistant KMS-11 myeloma cells. RID-F conjugated to hydrophobic peptides also inhibited the 26S proteasome but failed to induce cell death, suggesting poor penetration into cells. We infer that the R_8 peptide has dual functions: (1) rapid penetration of conjugates into the cell increases intracellular drug concentrations sufficient for exhibition of its effect, and (2) recognition of the conjugates by the 26S proteasome stimulates drug entry into the catalytic chamber. In the presence of ATPγS, RID-F conjugates containing R_8 inhibited the 26S proteasome more potently than in the presence of ATP, suggesting efficient entry of drugs into the catalytic chamber in a similar fashion to the substrate. Taken together with docking simulations of RID-F conjugate interactions with proteasome active sites, the second function of R_8 peptide is plausible. Thus, the conjugation of nonpeptidic proteasome inhibitors to a cell-penetrating peptide could represent a viable strategy for overcoming the drug-resistance of tumor cells.

1. Introduction

In eukaryotic cells, protein degradation by the ubiquitin-proteasome system plays a pivotal role in the regulation of various cellular processes including proliferation, differentiation, apoptosis, gene transcription, signal transduction, and quality control of nascent proteins [1]. The 26S proteasome, which is constitutively present in eukaryotic cells, comprises a barrel-shaped core particle (20S proteasome) and the 19S regulatory particles (RPs) capping one or both ends of the 20S proteasome barrel [2–4]. The 20S proteasome consists of seven α-subunits (α1–7) and seven β-subunits (β1–7) that form respective heptameric ring structures (α-ring and β-ring). Two α-rings and two β-rings are stacked in an αββα arrangement, generating the barrel-like structure of the 20S proteasome. The proteolytic active sites are located inside the barrel, where the β1, β2, and β5 subunits are responsible for protease activities with distinct substrate specificities: β1 for peptidylglutamyl peptide hydrolase (PGPH), β2 for trypsin-like (T-L), and β5 for chymotrypsin-like (CT-L) activities [5,6].

The 19S RPs regulate translocation of substrates into the 20S proteasome barrel. There are two types of 19S RP sub-complexes, known as the lid and the base, which contain different subunits with different functions. Deubiquitylation and unfolding of substrates are required prior to translocation of substrates into the catalytic chamber. The lid removes ubiquitin from poly-ubiquitinated substrates [7,8], while the base unfolds the globular domains of substrates [9,10]. One of the components in the base, a

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heterohexameric ring with AAA-ATPase modules, plays a critical role in substrate translocation into the 20S proteasome barrel and substrate unfolding. ATP binding to the base opens the gate for substrates to be translocated into the barrel, and subsequent hydrolysis of the bound ATP is required for substrate unfolding mediated by reverse chaperone or unfoldase associated with AAA-ATPases.

Disruption of the ubiquitin-proteasome system causes accumulation of incompatible regulatory proteins that trigger an apoptotic cascade, eventually leading to growth arrest and cell death. In comparison with normal cells, tumor cells are generally more sensitive to the proapoptotic effects of proteasome inhibition [11]. This was ascertained in multiple myeloma cells whose growth strongly relies on the transcription factor NFκB [12,13]. Binding of IκB to NFκB in the cytoplasm prevents translocation of NFκB into the nucleus. Stress-induced phosphorylation of IκB results in proteasome-dependent degradation of IκB and thus NFκB activation. Inhibition of the proteasome likely represses myeloma cell growth by maintaining NFκB in an inactive state. Thus, proteasome inhibitors (PIs) have emerged as a new class of molecular-targeted anticancer drugs [14].

Several PIs are in clinical use as anticancer drugs [13,14]. One inhibitor, the peptide boronate bortezomib, is approved for the treatment of multiple myeloma and mantle cell lymphoma [15,16]. The toxic boronate pharmacophore, however, is associated with severe adverse effects, which is why an epoxomicin analog, tetrapeptide epoxyketone carfilzomib, was developed and approved [17]. More recently, another peptide, boronate ixazomib, which is available for oral uptake, has been approved for the treatment of multiple myeloma in combination with two other drugs, lenalidomide and dexamethazone, after first-line therapy [18]. In addition to the treatment of hematological malignancies, PIs have been proposed as promising drug candidates for solid tumors, inflammation, immune diseases, ischemic stroke, and tuberculosis [19]. This situation encourages the development of new types of PIs with improved efficacy and fewer adverse effects [20].

To find new noncovalent and nonpeptidic PIs, we previously examined proteasome inhibition using a series of tamoxifen derivatives and found that ridaifen (RID)-A, -B, -D, and -F inhibited protease activities of the 20S proteasome [21]. RID-F (**1**) was the most potent inhibitor of the three different enzymatic activities. However, subsequent investigation indicated that RID-F derivatives were not effective against multidrug-resistant human multiple myeloma cells (KMS-11). Drug resistance can be caused by poor drug uptake, active excretion, intracellular degradation or modification, concentration of drugs into a specific subcellular compartment, and mutations that render target proteins insensitive to drugs. Although it is difficult to identify the mechanisms that give rise to drug resistance in individual cells, it is likely that poor drug uptake or active excretion is a prevalent mechanism, and therefore modification of drugs to improve uptake is a promising strategy.

Several proteins, such as Tat protein from human immunodeficiency virus-1 (HIV-1), can spontaneously penetrate cells [22]. The minimal domain needed for penetration was defined as a short sequence of 10–16 amino acids, termed a "cell-penetrating peptide" (CPP) [23]. Native Tat peptide is composed of several cationic amino acids, including six arginine and two lysine residues. Furthermore, the cationic peptide, octaarginine (R_8), can promote cellular uptake of conjugated compounds. Although several hypotheses explaining how these peptides penetrate cells have been presented [24,25], the underlying mechanism still remains unclear. Nevertheless, CPPs can be used for the translocation of peptides, proteins, ribonucleic acids, oligonucleotide mimics, and nanoparticles through cell membranes. Since CPPs can also transport nonpermeable drugs into living cells, they are of considerable pharmaceutical interest. Studies of conjugating CPPs to several drugs, including methotrexate [26], doxorubicin [27–31], and organometal conjugates [32–34], have been reported. Tat peptide conjugated to doxorubicin significantly increases the intracellular concentration of the drug in both drug-sensitive and drug-resistant cell lines [29].

In this study, we prepared conjugates of RID-F and various peptides, including oligo-arginines, and compared their inhibitory potency on the protease activities of the 20S and 26S proteasomes. Notably, RID-F conjugates containing R_8 showed a strong inhibitory potency toward 26S proteasome activity in comparison with that of free RID-F. Furthermore, these RID-F conjugates overcame KMS-11 cell drug resistance, rapidly inducing cell death. We confirmed that these conjugates inhibited proteolytic activities of the intracellular proteasome. These results suggest that conjugation of nonpeptidic PIs with CPPs is a viable strategy to overcome tumor-cell drug resistance.

2. Results

2.1. Synthetic procedures

We previously presented the synthesis and characterization of a novel tamoxifen derivative, RID-F (**1**), which inhibits 20S proteasome activity. RID-F was synthesized chemically as described in previous reports [21,35,36]. RID-F derivatives (RID-F-COOH (**15**) and RID-F-CH_2NH_2 (**19**)) were prepared by the Mukaiyama reductive coupling reaction [37,38], as shown in Schemes 1 and 2. The 1,3-phenylenedimethanol (**2**) was first mono-protected with a *p*-methoxybenzyl group and the following two-carbon elongation of the intermediate **4** to afford 1-(3′-((*p*-methoxybenzyloxy)methyl) phenyl)propan-1-one (**6**). Then, **6** was treated with an excess amount of 4,4′-dihydroxybenzophenone (**7**) in the presence of the low-valent titanium species generated from titanium(IV) chloride with zinc powder to afford the desired cross-coupling product **8** in 52% yield. The phenol moieties in **8** were temporarily protected as *tert*-butyldimethylsilyl (TBS) groups and finally transformed into the corresponding aminoethyl ethers by alkylation of **14**. Fortunately, the benzyl protective group in the tetra-substituted olefin **14** was simultaneously cleaved in the alkylation reaction under nucleophilic conditions to provide the desired RID-F derivative **15** including the free carboxylic acid part.

Next, RID-F derivative **19**, including the free primary amine part, was also synthesized using a similar procedure as described in Scheme 2. Aminoethyl groups were introduced into the bisphenol **17** in 77% yield, and the desired free primary amine **19** was successfully prepared from benzyl oxime **18** by reduction using lithium aluminum hydride in 77% yield.

Peptides and the RID-F derivatives were linked at the N- or C-terminus of the peptides by amide bond formation (Scheme 3). In the peptides used for the conjugation, the opposite terminus to the site linked with the RID-F derivatives was blocked by modification with an amide (C-terminus) or acetyl group (N-terminus). The chemical structures of representative compounds, RID-F (**1**), RID-F-GR_8 (**27**), and R_8G-RID-F (**31**), are depicted in Fig. 1. The purities of all the conjugates were confirmed by analytical reversed-phase high-performance liquid chromatography (RP-HPLC) analysis and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass measurements, and were above 95% (Supplementary Fig. 4). The *m*/*z* values of these compounds were observed to be identical with the theoretical values (Supplementary Table 1).

2.2. Proteasome inhibition by RID-F-CPP conjugates

To assess the potency of proteasome inhibition of RID-F (**1**) and