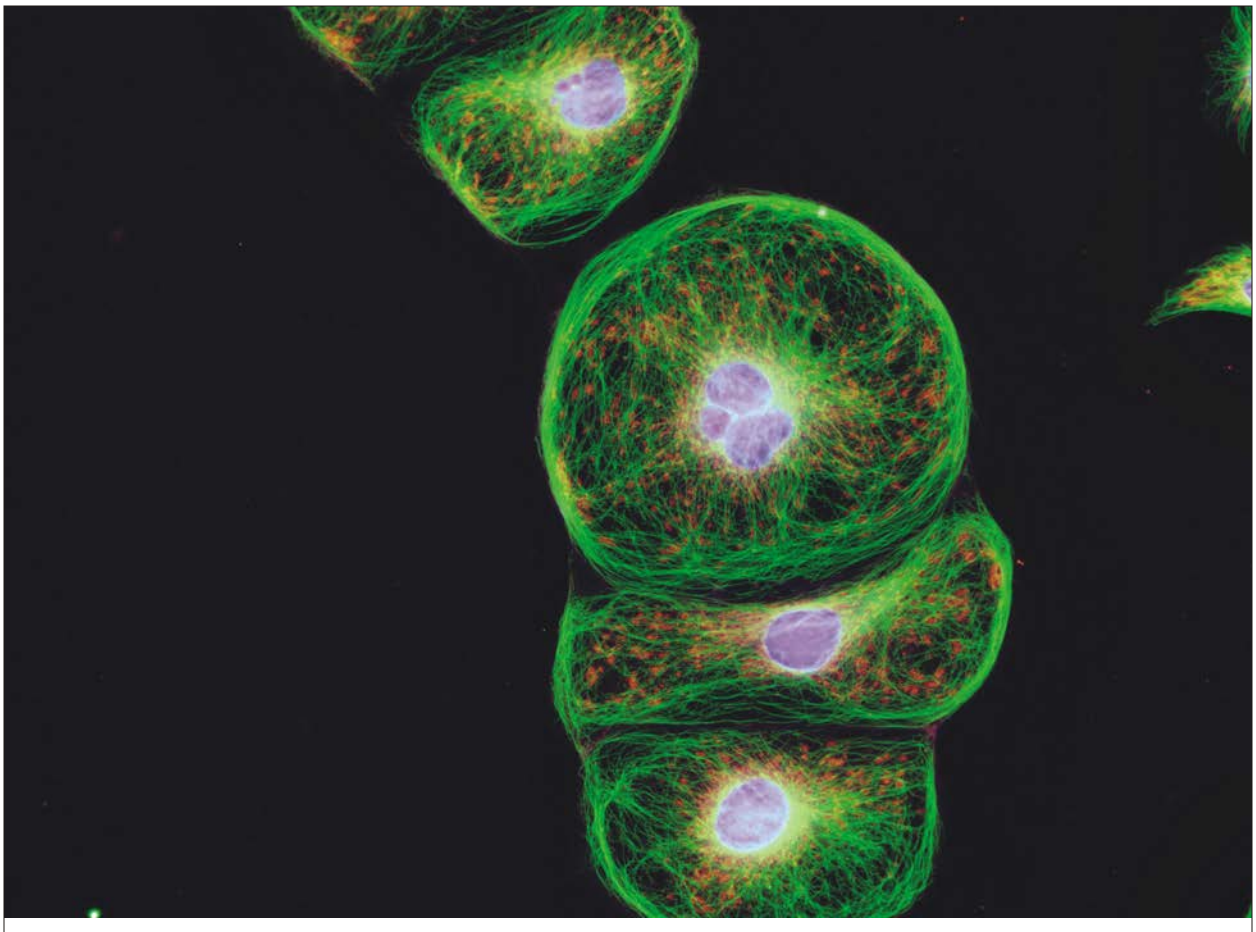

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A Mini Review on New Pharmacological and Toxicological Considerations of Protease Inhibitors' Application in Cancer Prevention and Biological Research

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ABSTRACT

Proteases are enzyme complexes that play a crucial role in the degradation of many proteins involved in regulation of cell cycle including G_1, S and G_2/M phases, apoptosis, cells growth and activation, adhesion, invasion, cell migration and metastasis, protein secretion, cellular interactions and signal transduction, phagocytosis and angiogenesis as well as tissue formation and stabilization. Thus, they show complete anticancer mechanisms. Proteases may be classified by their catalytic mechanisms into aspartic, serine, threonine, metallo- and cysteine proteases and are localized at the cellular surface or within specific sub cellular structures, in particular the lysosomes. *In vitro* and *in vivo* studies have reported the anticancer properties of protease inhibitors (PIs) but little is known about the clinical use of natural or chemical PIs, alone or in combination with other chemotherapies in humans. Thus, understanding the mechanisms of these drugs in prevention of cancer could result in them being used for treating the diseases. Furthermore, PIs can attenuate the drug toxicity in studies. They can also promote the defense mechanism, including induction of superoxide dismutase (SOD) and catalase (CAT) activity. These properties, on the one hand, suggest a wide spectrum of clinical applications for treatment and prevention of various cancers and on the other hand, further clinical and biological studies need to know the accordance mechanisms and discover new natural PIs. Thus, studying PIs will open a new anticancer strategy in treatment of various cancers in future.

Key words: Cell cycle regulation, apoptosis, natural and chemical protease inhibitors, cancers, toxicity

INTRODUCTION

PIs are based on special protease types in which inhibitors are classified into aspartic protease, cysteine protease, metalloprotease, serine protease (serpins), threonine protease, trypsin and Kunitz STI protease inhibitors (Rawlings *et al*., 2004). PIs are localized at the cellular surface or sub

cellular structures, in particular the lysosomes. PIs act as a crucial element in various physiological reactions from simple digestion of food proteins to highly-regulated event including immune system, angiogenesis, apoptosis pathways and cell growth, differentiation, vasculogenesis, as well as effecting cell migration and metastasis which are important in developmental and repair processes (Buhling *et al*., 2006).

PIs were commonly used to treat viral infection, including HIV (Chow *et al*., 2009; Danwe *et al*., 2005; Judith *et al*., 2007; Van Heeswijk *et al*., 2001) and hepatitis C for a long time (Nelson, 2011; Ghosal *et al*., 2011). PIs can inhibit the viral development by preventing the production of proteins which are important to assemble new virus particles (virions) (Chow *et al*., 2009; Van Heeswijk *et al*., 2001).

PIs have been recognized as a special approach in anticancer therapy and mostly can be isolated from plants (Rahimi *et al*., 2011; Khan *et al*., 2008; Tochi *et al*., 2008) or bacteria (Yadav *et al*., 2010; Hossain *et al*., 2007) as their natural sources. Studies *in vitro* and *in vivo* have reported that PI compounds have anticancer properties on their own or synergistically with other compounds. In one study a combination of natural compounds such as gambogic acid with PIs, including MG132 or MG262 (carbobenzoxy-L-leucyle-L-leucyl-L-leucinal), have shown inhibitory effects on growth of malignant cells and tumors in allograft animal models with no observed systemic toxicity in the animals (Huang *et al*., 2011). PIs have been shown to have a potent anticancer effect against various cancers like leukemia, lymphomas, melanoma, hepatocellular carcinoma, prostate, lung, breast, ovarian, cervical, colorectal and gastric cancer (Table 1, 2). Different cancer cell lines have been used for evaluating the anticancer effect of PIs (Table 1, 2). A PC3 prostate cancer cell line is one of the examples in which multiple death signaling pathways can be regulated by PIs (Yang *et al*., 2006). Thus, PIs represent a complete anticancer effect. Furthermore, except potential attenuation of chemotherapeutic drug toxicities properties, they have proved to be beneficial anticancer drugs, alone or in combination with other anticancer drugs, in new treatment strategy.

Anticancer properties of chemical protease inhibitors: Cancer drugs development is slow and costly. Therefore, an approach to accelerate the availability of new drugs is to reposition drugs approved for other indications as anticancer agents. Studies are investigating whether PIs could possibly be used to treat cancers. They are, also, evaluating its position in future. PI drugs such as nelfinavir and atazanavir are able to kill tumor cells *in vitro* (Gills *et al*., 2007; Pyrko *et al*., 2007) and *in vivo* (Pyrko *et al*., 2007), however, this effect has not been completely tested in humans yet. Therefore, wide spectrum research is necessary to be designed according to PIs in human to evaluate its beneficial role. PIs may be targeted for p-glycoprotein 1 (P-gp) also known as multidrug resistance protein 1 (MDR1) or cluster of differentiation 243 (CD243) or ATP-binding cassette sub-family B member 1 (ABCB1), but they interestingly have interaction with P-gp and can inhibit its effect and remain in cells for a long time or they may inhibit the effluxes of other anticancer drugs for more effect and can potentiate its anticancer properties (Washington *et al*., 1998; Olson *et al*., 2002). Ritonavir has been reported as the modulation effect on pharmacokinetic (PK) properties of mirtazapine and citalopram and can increase the peak of these two antidepressants in mice, then Therapeutic Drug Monitoring (TDM) have been necessary when these combinations are needed (Thakar *et al*., 2012). Inhibitors of protease, can now front-line drugs for the treatment of various cancers in human. Table 1 shows the special considerations for chemical PIs in cancer treatment. Anti metastasis and invasion, cell cycle regulation, differentiation

Table 1: Chemical protease inhibitors and their probable anticancer mechanisms in cancer cell lines

Studies	Cell lines	Cancer types	Probable anticancer mechanisms	Chemical protease inhibitors
Timeus *et al.* (2006)	K562, K562-R and KCl22-R	Leukemia	NFkB activation and degradation of IkB effect	Saquinavir
Rothweiler *et al.* (2010)	C6 and B16	Glioma and melanoma	Blocking the up regulation of p53 expression and reducing the differentiation of C6 glioma and B16 melanoma growth	Saquinavir
Donia *et al.* (2011)	PC-3	Prostate cancer	Enhancing the production of proapoptotic Bim and restoration of tumor necrosis factor (NF-βB) apoptosis	Saquinavir-Nitric Oxide (NO)
Gupta *et al.* (2005)	SQ20B, T24, MIAPACA2 and A549	Lung cancer	Viability decreasing in tumor cells via inhibiting phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathway	Amprenavir, Nelfinavir and Saquinavir
Gills *et al.* (2008)	A549, H460 and MCF7	Lung and breast cancer	Reducing the growth factor receptor activation causing Akt signaling and up regulates markers of endoplasmic reticulum (ER) stress, autophagy and caspase-dependent apoptosis	Nelfinavir
Srirangam *et al.* (2011)	A549, H522, H23 and K-ras wild-type line H838	Lung and breast cancer	G0/G1 cell cycle arrest and induction of apoptosis, down-regulation of cyclin-dependent kinases (cyclin D1) and retinoblastoma protein phosphorylation and inhibition of survivin messenger RNA and protein levels	Ritonavir
Kumar *et al.* (2009)	MDH-2774 and SKOV-3	Ovarian cancer	G1 cell cycle arrest, inhibition of AKT signaling, activates apoptosis and inhibits migration and invasion	Ritonavir
Hampson *et al.* (2006)	SiHa, CaSki, C33A, C33A-E6 and non-transformed NIH/3T3 cells	Cervical carcinoma	Inhibiting of p53 degradation	Lopinavir and Indinavir
Srirangam *et al.* (2006)	MCF7, T47D, MDA-MB-436 and MDA-MB-231	Breast cancer	G1 cell cycle arrest, depletes cyclin-dependent kinases 2, 4 and 6 and cyclin D1 but not cyclin E and depletes phosphorylated Rb and Ser 473 Akt	Ritonavir
Ikezoe *et al.* (2006)	HL-60 cells	Myeloid leukemia	Potentiates the effect of 1,25-dihydroxyvitamin D3 to induce growth arrest and differentiation of human myeloid leukemia cells through down-regulation of cytochrome p450 (CYPs) 24 enzymes	Ritonavir
Bruning *et al.* (2011)	SiHa	Cervical cancer	Change in the expression of cell cycle-regulatory cyclins, induction of cell cycle arrest and unfolded protein response (UPR) and caspase apoptosis	Nelfinavir
Libertini *et al.* (2007)	LNCaP	Prostate tumors	Inhibition of calpain activity which responsible for cleaves the androgen receptor (AR) into an androgen-independent isoform and can promote apoptosis in androgen-independent Rv1 cells	Amprenavir
Pyrko *et al.* (2007)	U251 and LN229	Malignant glioma	Stimulation of endoplasmic reticulum (ER) stress response (ESR)-associated proapoptotic caspase-4	Nelfinavir and Atazanavir

Table 2: Natural protease inhibitors and their probable anticancer mechanisms in cancer cell lines

Studies	Cell lines	Cancer types	Probable anticancer mechanisms	Constituents	Plants
Li *et al*. (2011)	HCT116 and SW480	Colorectal cancer	Cell death caused by mediating caspase dependent apoptosis and cytoplasmic vacuolation. Activation of p53, Bax and NF-βB pathway occured in accordance to increase in (reactive oxygen species) ROS levels and BCL2 reduction	Ginsenosides	American ginseng
Dai *et al*. (2010) Liu *et al*. (2011)	PC-3, DU145 and CL1	Prostate cancer	NF-βB activity attenuation, also G1 cell arrest, caspase activation and PARP cleavage is occurred	Diterpenoid epoxide triptolide and the quinone triterpene celastrolare	Chinese Thunder of God vine (T. wilfordii).
Shankar and Srivastava (2007) Bachmeier *et al*. (2010)	MCF7	Breast cancer	Reducing in breast cancer and menopausal symptoms by estrogen like effect to compete with endogenous estrogen; Caspase dependent apoptosis and Bax and Bak regulation	Curcumin, Quercetin and Enterolactone	Turmeric (Curcuma longa)
Cheng and Su (2010) and Liu *et al*. (2009)	HCC J5 and THP-1	Hepatocellular carcinoma and leukemia	Increasing in p53, p21, Bax, calreticulin, caspase 12, caspase 3 and GADD153 expression were occurred, but Bcl-2, Cdc2 and Cdc25c were decreased	Tanshinone IIA (Tan-IIA)	Danshen (Salviae miltiorrhizae Radix)
Xu *et al*. (2010)	Ls174-T	Colon carcinoma	Anti cancer metastasis activity through extracellular signal-regulated kinase pathway, including inhibition the expression of metalloproteinase-2 and 9	Prunella vulgaris L.	Rosmarinic acid
Lou *et al*. (2010)	MGC-803	Gastric cancer	Increasing of caspase-3 activity and decreasing of survivin mRNA expression	Herba Oxytropis	2',4'-Dihydroxychalcone (TFC)
Shin *et al*. (2009)	DU145	Prostate cancer	Inhibition of signal transducer and activator of transcription 3 (STAT3) and its downstream protein expression cyclin D1, survivin and Bcl-xL	Salvia miltiorrhiza Bunge	Cryptotanshinone

and apoptosis of cancer cells and related protein expression may be affected by PIs. These anticancer mechanisms emphasize the complete anticancer properties of PIs which represent a potential strategy for the treatment of human cancers in the future. Other PI drugs and accordance anticancer mechanisms *in vitro* are presented in Table 1.

Anticancer properties of natural protease inhibitors: Different cellular events, including DNA repair in cell cycle and survival and apoptosis including p27, a key regulator for G1 to S transition in the cell cycle, can be regulated by natural drugs or their consistent (Yang *et al*., 2009). In one study, pro-apoptotic regulator agent BAX has been increased by Shikonin, a natural naphthoquinone of Chinese medicine Zi Cao (gromwell) (Yang *et al*., 2009) and decreased in the levels of anti-apoptosis regulator agent Bcl-2 in another study (Sohrabi *et al*., 2009; Dai *et al*., 2010). Changes in the activity of nuclear factor kappaB (NF-κB), an important inflammatory agent (Hemalatha *et al*., 2012; Miroliaee *et al*., 2011; Arathy and Sreekumar, 2009) and pro-survival factor that is pivotally regulated by the proteasome, has been reported by natural drugs (Rahimi *et al*., 2010).

5,6,3',4'-tetrahydroxy-7-methoxyflavone, a novel natural PI which is known for its anticancer effect, is extracted from *Anisomeles ovata* (Chang *et al*., 2010). Celastrol is another natural PI that exhibits promising anticancer property by inhibiting the constitutive NF-κB activity and modulating the Bcl-2 family proteins, suppressed cell migration and invasion, increasing caspase dependent apoptosis and decreasing angiogenesis in androgen-independent prostate cancer cell lines PC-3, DU145 and CL1 (Dai *et al*., 2010). Bowman-Birk protease inhibitor which is obtained from *Vigna unguiculata* seeds has shown an inhibitory effect on trypsin and chymotrypsin, two enzymes belonging to the serine family of proteases in which regulated the immune function, blood clotting and inflammation (Joanitti *et al*., 2010; Srinivasan and Durairaj, 2007). It has been suggested that this preparation reduces cancer cells' ability to duplicate and spread within 72 h, leading to apoptosis or cell death anticancer effect against MCF-7 breast cancer cells by reducing this cell viability and proliferation through S and G2/M phase arrest (Joanitti *et al*., 2010). Furthermore, DNA fragmentation, mitochondrial protection and cytoplasm acidification were also reported (Joanitti *et al*., 2010). Study has indicated that a type of blue-green algae known as *Microcystis aeruginosa* may be a potential new source of natural protease inhibitors containing two previously unknown inhibitors of the trypsin protease, including micropeptins EI992, EI964 and a modified linear peptide aeruginosin EI461 which is isolated from its hydrophilic extract (Ploutno *et al*., 2002). Green tea which grows in the north of Iran and has proved to be hepatoprotective and antioxidant, contains a type of polyphenol called epigallocatechin gallate (EGCG) which has shown PI and anticancer activities against human breast cancer cells (Balouchzadeh *et al*., 2011; Dou *et al*., 2008). Ellagic acid and punicalagin, from *Punica granatum* (pomegranate) have been also reported an inhibitory effect on serine PI, including alpha-secretase (TACE), chymotrypsin, trypsin and elastase (Kwak *et al*., 2005). Catechin, quercetin, kaempferol, equol and epicatechin (epigallocatechin-3-gallate) are the other constituents of pomegranate which may also have protective effect (Park *et al*., 2010). Different anatomical compartments of pomegranate, including seed, juice, peel, leaf, flower, bark and roots may have anticancer activity through counteraction with tumor cell proliferation, invasion, cell cycle and angiogenesis (Lansky and Newman, 2007; Rahimi *et al*., 2011). Pineapple (*Ananas comosus*) is another plant which contains PIs and can be used for its therapeutic properties including inhibition of malignant cell growth, inflammation, thrombus formation, control of diarrhea, dermatological and skin

debridement (Tochi *et al*., 2008). Collectively, further studies are necessary to be arranged for detection of new natural PIs in plants and recognizing the accordance anticancer mechanisms. Thus, working on natural PIs will be dispread in the world, because of their low toxicity and cost, good response and anticancer properties. Other reported anticancer mechanisms of natural PI *in vitro* are shown in Table 2.

Toxicological overview of protease inhibitors: Further, beneficial effects of PIs are in accordance with reduction in toxicity of anticancer or chemotherapeutic drugs in combination. Indinavir, nelfinavir, saquinavir and ritonavir are currently PIs which are administrated to ameliorate doxorubicin induced cardiomyopathy which are mediated by stimulation of Toll-Like Receptors (TLR) 2 and 4 expression on cardiomyocytes (Kast *et al*., 2007). Combination of PI bortezomib/PS-341 in multiple myeloma cells refractory with multiple prior therapies, including melphalan, dexamethasone and thalidomide can reduce drug resistance and attendant toxicity and improve patient outcome in multiple myeloma (Chauhan *et al*., 2005). PIs may be considered much because of a better bio-distribution, lower toxicity and inhibitory quality on matrix metalloproteinases (MMP), indicator factors of metastases and the proteasome for a more effective anticancer therapy (Toschi *et al*., 2011). Furthermore, reduced glutathione, a major defense mechanism against Reactive Oxygen Species (ROS) (Nili-Ahmadabadi *et al*., 2011) has been restored by rosmarinic acid, a main phenylpropanoid constituent of *Prunella vulgaris* L. PIs' other protective roles are served as the reducer of Malondialdehyde (MDA) level and liver enzyme activity of aspartate aminotransferase (AST), alanine aminotransferase (ALT), as well as a lactate dehydrogenase (LDH) which can be induce by alcohol or other poison. Increase in the level of anti oxidative mechanisms, including superoxide dismutase (SOD) and catalase (CAT) activity (Polat *et al*., 2008). Furthermore, inflammatory cytokines which are important in induction of various diseases (Kalani *et al*., 2011; Rahimi *et al*., 2010), have been decreased with aprotinin (Homi *et al*., 2010). PIs may also be considered much more from the perspective of toxicology and have beneficial properties in comparison to other anticancer drugs.

DISCUSSION

PIs represent an important role in regulation of various cellular physiological and biological processes, including cell cycle, cell death, differentiation and the immune response (Fan *et al*., 2001; Buhling *et al*., 2006). Previous studies have shown the anticancer effect and probable mechanisms of the natural and chemical PIs *in vitro* (Table 1, 2).

PIs, like MG132, have shown apoptosis effects against gastric cancer cell lines AGS (p53 wild-type) and MKN-28 (p53 mutant) through a time and dose-dependent stimulation of caspase-3 which results in the release of cytochrome C from mitochondria into the cytosol, as a consequence of up-regulation of BAX. Furthermore, over-expression of all protease-associated proteins, including p53, p21 (waf1) and p27 (kip1) were demonstrated 4 h after protease inhibition. These results indicate a potential effect of PIs as anticancer drugs in gastric cancer (Fan *et al*., 2001). Two novel analogs, CH05-0 and CH05-10 of Indinavir, a Human Immunodeficiency Virus (HIV) protease inhibitor, inhibits the growth of cancer cells *in vivo* by induction of G_1 cell cycle arrest, caspase-dependent apoptosis, stimulation of endoplasmic reticulum stress and unfolded protein response. In addition, no cytotoxic effect was observed against cancer cells *in vitro* (You *et al*., 2010). Also, acetyl salicylic acid (aspirin) induces synthesis of maspin, a member of the serine protease inhibitor which has been shown to inhibit the incidence of breast cancer

metastasis and invasion in patients who have previously received anticancer therapies (Bhattacharyya *et al*., 2010).

A 17.5-kDa PI isolated from Chinese mini-black soybeans can inhibit HIV-1 reverse transcriptase (IC (50) = 3.2 and 5.5 μM), proliferation of breast cancer cells (IC (50) = 9.7 and 3.5 μM) and hepatoma cells (IC (50) = 35 and 6.2 μM), with relatively high potencies (Ye and Ng, 2011). Furthermore, PIs show trypsin inhibitory activity which is involved in carcinogenesis and promotes proliferation, invasion and metastasis of tumors and are found in various cancers (Ye and Ng, 2011; Soreide *et al*., 2006). Co-expressed MMP-2, MMP-7 and MMP-9 with trypsin seem to be of particular importance in proliferation, progression and invasion. In addition, MMPs are zinc-dependant endopeptidase which may play a role in both conversion from adenoma to carcinoma and in the initiation of invasion and metastasis (Wongsawatkul *et al*., 2011; Soreide *et al*., 2006; Xu *et al*., 2007). MMP and proteinase-activated receptor-2 (PAR-2) which are stimulated by trypsin, may activate the mitogenic MAPK-ERK pathway through activation of the epidermal growth factor receptor (Soreide *et al*., 2006). Such results have shown the molecular mechanisms of proliferation, invasion and metastases that are concerned with the role of trypsin in cancers consisting of colorectal cancer. Thus, PIs which have trypsin inhibitory activity may reduce cancer progression and may be regarded as a potential target of therapy. PIs can be also targeted for treatment of other diseases such as diabetes (Naderi *et al*., 2006).

PIs, as modulators in chemotherapy, may have adverse effects, on the one hand, through inhibition of worse events, including inflammatory cytokine production and liver enzyme activity and on the other hand, through promotion of protective mechanisms against oxidative stress, including superoxide dismutase (SOD) and Catalase (CAT) induction has been shown in accordance to PIs occupation. Furthermore, change in a pharmacokinetic event to the beneficial way like down regulation of P450 to induce active toxic metabolite is the other protective mechanism in which mediated by PIs (Ikezoe *et al*., 2006).

CONCLUSION

In numerous studies, the role of natural and chemical PIs in cancers have been reported *in vitro* and *in vivo*, but there is little information about the use of these compounds in inhibiting cancer in humans and the mechanisms of their actions. Thus, it will be a wide open spectrum of biological and clinical studies. Clinical trials, involving the use of these compounds, will be necessary in the future. Therefore, we propose that by making sure these compounds are devoid of toxicity, they can be used as potent anticancer drugs in the future.

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