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Effects of a novel carbocyclic analog of pyrrolo[2,3-*d*]pyrimidine nucleoside on pleiotropic induction of cell death in prostate cancer cells with different androgen responsiveness

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ABSTRACT

Prostate cancer is the most frequently diagnosed cancer and is one of the leading causes of male cancer death in the world. Recently, in the course of our screening for a novel anticancer compound, we synthesized carbocyclic analogs of pyrrolo[2,3-*d*]pyrimidine nucleoside; compounds **5**, and **6**. In the current study, we report the effects of compound **5** on pleiotropic induction of cell death via up-regulation of AR-associated p21^{Cip1} protein in prostate cancer cells with different androgen responsiveness, such as LNCaP (androgen-dependent and -sensitive), LNCaP^{C4-2} (androgen-independent and -sensitive; androgen-refractory), and DU145 (androgen-independent and -insensitive) cells.

The treatment of LNCaP cells with 6 μM compound **5** for 24 h stimulated the androgen receptor (AR) activity and dramatically up-regulated transcription (56-fold) of *p21^{Cip1}*, which, in turn, induces typical apoptosis in the cells. However, induction of apoptosis through up-regulation (23-fold) of AR-associated *p21^{Cip1}* achieved in LNCaP^{C4-2} cells was possible by intensive cell treatment with compound **5** (9 μM, 48 h), because the cells are less sensitive and independent to androgen than LNCaP cells. Furthermore, 6 μM compound **5**-treated DU145 cells, which exhibit extremely low AR activation due to no androgen responsiveness and dependency, showed neither up-regulation of p21^{Cip1} nor apoptotic induction. Instead, a different type of cell death, autophagy-like death through the LC3B-associated autophagosome formation, was obviously induced in DU145 cells.

Taken together, our results suggest that pleiotropic induction of prostate cancer cell death by compound **5** is determined by how efficiently and how abundantly androgen-dependent activation of the AR occurs, whereas compound **6** shows no induction of apoptosis in LNCaP cells.

Prostate cancer is the most frequently diagnosed cancer in men and is one of the leading causes of male cancer fatalities worldwide.[1,2] Prostate cancers are extremely heterogeneous in terms of biological, hormonal, and molecular characteristics, because they consist of both androgen-sensitive, and -insensitive cells. In general, prostate cancers can be treated by surgery, radiation and hormonal therapy.[3] Most of all, androgen deprivation therapy (ADT) can trigger cell death of prostate cancer, and remains one of the primary treatment options for all prostate cancer patients. ADT targets activity of the androgen receptor (AR) by reducing available ligands, such as androgens. However, despite the initial efficacy of ADT, the advanced prostate cancer patients eventually develop resistance to this therapy and progress to hormone-refractory prostate cancer, for which there is no curative therapy.[4,5]

LNCaP cells described as "androgen-dependent" require androgens to grow and respond to physiological levels of androgen-responsive genes such as *PSA* (prostate-specific antigen) gene,[6] whereas, the classical prostate cancer cells, such as DU145, are identified as "androgen-independent" due to the extremely low level of the AR. In contrast, LNCaP^{C4-2} cells derived from LNCaP cells grow in the absence of androgens, yet respond to manipulation of androgen levels. Thus, LNCaP^{C4-2} cells are described as "androgen-independent", and also "androgen-sensitive", namely "androgen-refractory".[7]

The cytoplasmic AR is a member of the steroid hormone receptor superfamily and can act as a latent transcription factor in

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response to androgen.[1,3] After binding to androgens, the active AR complex translocate into the nucleus to induce expression of target genes, such as *PSA*, *CDK2* and *p21^{Cip1}*, which are involved in many cellular activities, from proliferation to apoptosis.[2,8–11] However, the cellular functions of p21^{Cip1} mediated by the AR on induction of cell death remain unknown.

Programmed cell death (PCD) and cell proliferation have been characterized as fundamental cellular events to maintain the tissue homeostasis of the organism. Therefore, an imbalance between cell death and survival through deregulated cell cycle progression and impaired cell death induction is involved in neoplastic cancer formation.[12] Since the first descriptions of PCD mechanisms, several attempts have been made to classify cell death modalities based on morphological characteristics, such as 'type I cell death' associated with apoptosis, 'type II' with autophagy and 'type III' corresponding to necrosis.[13–15]

Apoptosis is characterized by specific morphological and biochemical changes of dying cells, including cell shrinkage, nuclear condensation and fragmentation, dynamic membrane blebbing and loss of adhesion to the extracellular matrix.[14,15] Autophagy is an evolutionarily conserved catabolic process beginning with formation of autophagosomes, double membrane-bound structures surrounding cytoplasmic macromolecules and organelles, destined for lysosomal recycling.[16–19] In general, autophagy plays a crucial pro-survival role in cellular homeostasis, which is required during periods of starvation or stress due to growth factor deprivation.[20] However, there is accumulating evidence that autophagic cells may commit suicide by undergoing cell death, which differs from apoptosis and necrosis.[13,21]

Recently, in the course of our screening for a novel anticancer compound, we synthesized several carbocyclic analogs of pyrrolo [2,3-*d*]pyrimidine nucleoside, and reported its anticancer activities in human ovarian cancer PA-1 cells.[22] In the current study, we report the effects of compound **5** on pleiotropic induction of cell death via up-regulation of AR-associated p21^{Cip1} protein in prostate cancer cells with different androgen responsiveness, such as LNCaP (androgen-dependent and -sensitive), LNCaP^{C4-2} (androgen-independent and -sensitive; androgen-refractory), and DU145 (androgen-independent and -insensitive) cells.[23]

We synthesized two novel carbocyclic analogs of pyrrolo[2,3-*d*] pyrimidine nucleoside, compound **5** (4-amino-6-bromo-1-cyclopentyl-1*H*-pyrrolo[2,3-*d*]pyrimidine-5-carboxamide) and **6** (4-amino-6-bromo-7-cyclopentyl-7*H*-pyrrolo[2,3-*d*]pyrimidine-5-carboxamide) from tetracyanoethylene (Fig. 1). 4-Amino-6-bromo-5-cyanopyrrolo[2,3-*d*]pyrimidine (**2**) was synthesized by the reaction of 2-amino-5-bromo-3,4-dicyanopyrrole (**1**) with formamidine acetate in 2-ethoxyethanol. The reaction of intermediate **2** with cyclopentyl bromide in the presence of sodium hydride as a base produced the two regioisomers **3** and **4**. By oxidative hydrolysis of compound **3** and **4**, compound **5** and **6** were obtained, respectively.[22]

To investigate the effects of compound **5** on the cell death induction in human prostate cancer cells with different androgen responsiveness, we examined the morphological characteristics by transmission electron microscopy (TEM) analysis and the expression pattern of apoptosis-related proteins by Western blotting in DU145 and LNCaP cells treated with 0, and 6 μM compound **5** for 24 h.

The morphological characteristics for the apoptotic cells, such as chromatin condensations and the formation of crescent-shaped apoptotic bodies, were obviously found in LNCaP cells treated with 6 μM compound **5** (Fig. 2A). Furthermore, the proteolytic cleavage of poly(ADP-ribose) polymerase (PARP), an enzyme involved in DNA repair, and activation of procaspase-3 into caspase-3, as well as dramatic up-regulation of p21^{Cip1} protein were identified in compound 5-treated LNCaP cells (Fig. 2B). In contrast, we could not identify any apoptotic features in 6 μM compound 5-treated DU145 cells. As shown in Figure 2A, however, the treatment of DU145 cells with compound **5** resulted in the accumulation of numerous autophagic vacuoles in the cytoplasm. In general, the cleavage of PARP and the vacuole formation were used as typical indicators of the apoptotic and autophagic inductions, respectively, in response to drug treatment.[24–26]

Autophagy recognized as an essential function for adaptation to environmental stress, such as nutritional starvation and energy depletion,[18–21] occurs when the cytoplasmic content to be degraded is surrounded by a small portion of membrane, creating autophagosomes. This autophagosome is then fused to the lysosome, creating an autolysosome and resulting in the autophagic vacuole formation through lysosomal degradation of cellular components. At the initiating step in autophagy, in general, the microtubule-associated protein-1 light chain 3B (LC3B) is required for the formation of autophagosomes.[27]

To determine whether the autophagy marker LC3B protein is located in the cytosol of compound **5**-treated DU145 cells, we examined the cellular distribution of LC3B by immunocytochemical analysis using fluorescence microscopy. The numerous cytoplasmic LC3B converted into its autophagosome-membrane associated form was obviously found as the white arrow indicated (Fig. 2C).

These results indicate that compound **5** apparently induces autophagy-like cell death through the autophagosome formation in androgen-independent and -insensitive prostate cancer DU145 cells, but apoptotic cell death in androgen-responsive prostate cancer LNCaP cells.

Therefore, our results suggest that compound **5** induces the pleiotropic cell death in prostate cancer cell lines depending upon their androgen responsiveness. Apoptosis occurred in LNCaP cells was induced by activated AR-mediated up-regulation of p21^{Cip1}, whereas, in DU145 cells in which the level of AR activity is extremely low, stimulation by compound **5** at 6 μM was insufficient to trigger apoptosis through AR activation but LC3B-mediated autophagy was alternatively induced. This idea corresponds our observation that weak but evident up-regulation of p21^{Cip1} and PARP cleavage occurred when DU145 cells were treated with compound 5 for 48 h instead of 24 h (Fig. 2D).

Recently, it has been reported that autophagy and apoptosis could be coincident or antagonistic, depending on experimental context.[26] To clarify whether these 2 types of cell death induced in compound **5**-treated DU145 cells were coincidentally or consecutively correlated each other, we examined TUNEL assay in DU145 cells pre-treated with 5 mM 3-methyladenine (3-MA), an autophagy inhibitor. As a result, we identified that suppression of autophagic induction by 3-MA apparently attenuate induction of apoptosis in DU145 cells treated with 6 μM compound **5** for 48 h. Thus, we can conclude that two types of cell death, apoptosis and autophagy, induced in compound **5**-treated DU145 cells were not coincidentally but consecutively correlated each other (Fig. 2E).

To identify the underlying molecular mechanism of compound **5**-induced apoptosis via up-regulation of AR-associated p21^{Cip1} in LNCaP cells, an AR inhibitor, flutamide, was used to antagonize the AR activity in compound **5**-treated LNCaP cells. Western blot analysis demonstrated that inhibition of AR activity using 100 μM flutamide significantly down-regulated the expression of p21^{Cip1} and also suppressed the apoptotic induction, such as activation of caspases and PARP cleavage (Fig. 3A).

Furthermore, as shown in Figure 3B, analysis using semi-quantitative real-time PCR (*q*RT-PCR) demonstrated that *p21^{Cip1}* expression was up-regulated more than 56-fold in LNCaP cells treated with 6 μM compound **5**, whereas the mRNA level of *p21^{Cip1}* in pre-treated cells with 100 μM flutamide decreased up to 99%. Thus,