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The prodigiosins, proapoptotic drugs with anticancer properties

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Abstract

The family of natural red pigments, called prodigiosins (PGs), characterised by a common pyrrolylpyrromethene skeleton, are produced by various bacteria. Some members have immunosuppressive properties and apoptotic effects *in vitro* and they have also displayed antitumour activity *in vivo*. Understanding the mechanism of action of PGs is essential for drug development and will require the identification and characterisation of their still unidentified cell target. Four possible mechanisms of action have been suggested for these molecules: (i) PGs as pH modulators; (ii) PGs as cell cycle inhibitors; (iii) PGs as DNA cleavage agents; (iv) PGs as mitogen-activated protein kinase regulators. Here, we review the pharmacological activity of PG and related compounds, including novel synthetic PG derivatives with lower toxicity and discuss the mechanisms of action and the molecular targets of those molecules. The results reported in this review suggest that PGs are a new class of anticancer drugs, which hold out considerable promise for the Pharmacological Industry.

Keywords: Apoptosis; DNA damage; Cancer; Chemotherapy; Prodigiosin

1. Introduction

In 1888, Dr. William B. Coley (1862–1936), a prominent New York surgeon, stumbled across one of the most intriguing findings ever made in cancer research. Dr. Coley combined the cultures of *Streptococcus* sp. and *Bacillus prodigiosus* (called *S. marcescens*), and then sterilised them by either heat or filtration. The mixture was called mixed bacterial vaccines (now called the Coley's toxins). This therapy was used to treat tumours with fascinating results in tumours of mesodermal origin [1,2]. Although the biologically active substance in Coley's toxins is described as tumour necrosis factor (TNF), a cytokine that is induced in response to lipopolysaccharide (LPS) and causes cancer cell death [3,4], PG might be contained in Coley's toxin. In fact, in recent years new interest in PG and its derivatives has emerged among researchers.

PGs are a family of naturally occurring polypyrrole red pigments produced by a restricted group of microorganisms, including some *Streptomyces* and *Serratia* strains, characterised by a common pyrrolyldipyrrolylmethene skeleton (Fig. 1). PG, cycloprodigiosin hydrochloride (cPrG·HCl), metacycloprodigiosin, nonylprodigiosin and undecylprodigiosin (prodigiosin 25-C, UP) are all members of this family. PG was first isolated from *S. marcescens* in pure form in 1929. Its name, used by early researchers, was retained but the pigment was not characterised and its main structural features elucidated until 1934 [5]. As typical secondary metabolites, PG and related materials have no clearly defined physiological functions in the producing organisms. However, PG is a wetting agent that provides ecological advantages in bacteria dispersion [6,7]. PG family members have potent antimicrobial, antimalarial, immunosuppressive and cytotoxic activity [8–33]. Recently, an extensive chemical research programme was undertaken by D'Alessio and co-workers from Pharmacia & Upjohn, in order to obtain synthetic derivatives of PG [34–36] and identify more active and less

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Abbreviations: cPrG·HCl, cycloprodigiosin hydrochloride; ds, double strand; ERK, extracellular signal-regulated kinase; IC_{50}, inhibitory concentration 50%; Jak3, janus kinase 3; JNK, c-*jun* N-terminal kinase; MAPK, mitogen-activated protein kinase; PG, prodigiosin; pH_i, intracellular pH; PKC, protein kinase C; SAPK, stress-activated protein kinase; ss, single strand; UP, prodigiosin 25-C (undecylprodigiosin).

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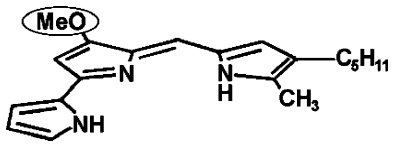

Fig. 1. Side-on view of 2-methyl-3-pentyl-6-methoxyprodigiosene (PG), showing the planar arrangements of the three pyrrole rings. The exceptional cytotoxic potency of PG may be attributed to the presence of the PG C-6 methoxy substituent (circle).

toxic drugs than natural PG compounds. The best compound obtained to date is PNU156804.

2. PGs trigger apoptosis

Apoptosis has become one of the newest areas of cell biology research, possibly because of the belated realisation that cell death is a biochemically regulated process that may be as complex as other fundamental biological processes. It has been linked to such diverse pathophysiologic processes as oncogenesis [36]. The activation of the apoptosis programme is regulated by various signals from both intracellular and extracellular stimuli. Indeed, in recent years evidence is beginning to accumulate that many (and perhaps all) agents of cancer chemotherapy kill tumour cells by launching the mechanisms of apoptosis.

New drugs associated with apoptosis are expected to be most effective against tumours with high proliferation rates and are being screened for use in the treatment of cancer [36]. Microbial pathogens engage or circumvent the host apoptotic programme. Indeed, PGs have been shown to induce apoptosis. Their apoptotic effects have been observed in several human cancer cell lines in tissue culture [19,24,29–33,37–39], in hepatocellular carcinoma xenografts [40] and in human primary cancer cells [33].

cPrG·HCl induces apoptosis in liver cancer cells both *in vitro* and *in vivo*, with high effectiveness on liver cancer and breast cancer cell lines, promyelocytic leukaemia cells and colon cancer cells [37–39], but nominally no toxicity on normal cells [39]. Apoptosis is the mechanism of action suggested for this molecule to exert immunosuppression [19,24]. However, PG rapidly and potently triggers apoptosis in haematopoietic cancer cell lines, including acute T-cell leukaemia, promyelocytic leukaemia, myeloma and Burkitt lymphoma cells [29]. PG also induces apoptosis in cells derived from other human cancers, including gastric [31] and colon [30], with no marked toxicity in non-malignant cell lines [29,31]. It also induces apoptosis of B and T cells in B-cell chronic lymphocytic leukaemia (B-CLL) samples [33].

Understanding the mechanism of action of PGs is essential for drug development and will require the identification and characterisation of their still unidentified cell target. Four possible mechanisms of action for these molecules have been suggested: (i) PGs as pH modulators; (ii) PGs as cell cycle inhibitors; (iii) PGs as DNA cleavage agents; (iv) PGs as mitogen-activated protein kinase (MAPK) regulators.

2.1. PGs as pH modulators

The pH within acidic organelles could be responsible for a wide variety of important cell functions, such as endocytosis, exocytosis and intracellular trafficking, as well as cell differentiation, cell growth and cell death [41]. It has been argued that the apoptotic process is modulated or triggered by changes in intracellular pH (pH_i) [42,43]. A very early event in mitochondria-dependent apoptosis involves a change in cellular pH regulation that is characterised by mitochondrial alkalinization and concomitant cytosol acidification [44]. Alteration of pH regulation precedes cytochrome *c* release from mitochondria and facilitates cytochrome *c* activation of caspases [44]. F-ATPase and V-ATPase inhibitors prevent changes in cytosolic pH and impair caspase activation and thus apoptosis [44]. Part of the action of PGs depends on their ability to uncouple vacuolar H^+-ATPase (V-ATPase) through promotion of the H^+/Cl^- symporter and to induce neutralisation of the acid compartment of cells, so bringing about intracellular acidification and eventually apoptosis (Fig. 2, Route 2) [37,38,40,45].

cPrG·HCl is a protonophore that raises lysosomal pH by inhibiting the proton pump activity of V-ATPase without affecting its ATPase activity [16]. Moreover, in the inhibition of vacuolar acidification by cPrG·HCl, Cl^- is required to collapse the chemical gradient of H^+ across the tonoplast [46]. In human breast cancer cells, which overexpress V-ATPase and maintain a higher pH_i than non-cancerous cells, cPrG·HCl inhibits the acidification of lysosomes, decreases pH_i and causes apoptosis. This suggests that high pH_i is necessary for the maintenance of the function of cancer cells, which are more sensitive to pH changes than normal cells [37]. Other studies in human promyelocytic leukaemia cells (HL-60) and in colon cancer cell lines support this hypothesis [38,39].

PG, metacycloprodigiosin and UP display H^+/Cl^- symport activity on liposomal membranes and uncouple both V- and F-ATPases, although they do not inhibit catalysis or membrane potential formation [45,47]. Additionally, UP induces functional and morphological changes in the Golgi apparatus and swelling of mitochondria [48].

Fürstner *et al.* reported that three pyrrole units are required for PGs to inhibit vacuolar acidification. They used two PG derivatives that essentially affect only one of these two biological responses: the proliferation of murine spleen cells or activity inhibiting vacuolar acidification. Thus, the action of PGs to inhibit proliferation is caused by mechanisms other than the inhibition of vacuolar acidification [26].

It would be useful to explore whether the drugs that modulate pH in cells through their effects on specific

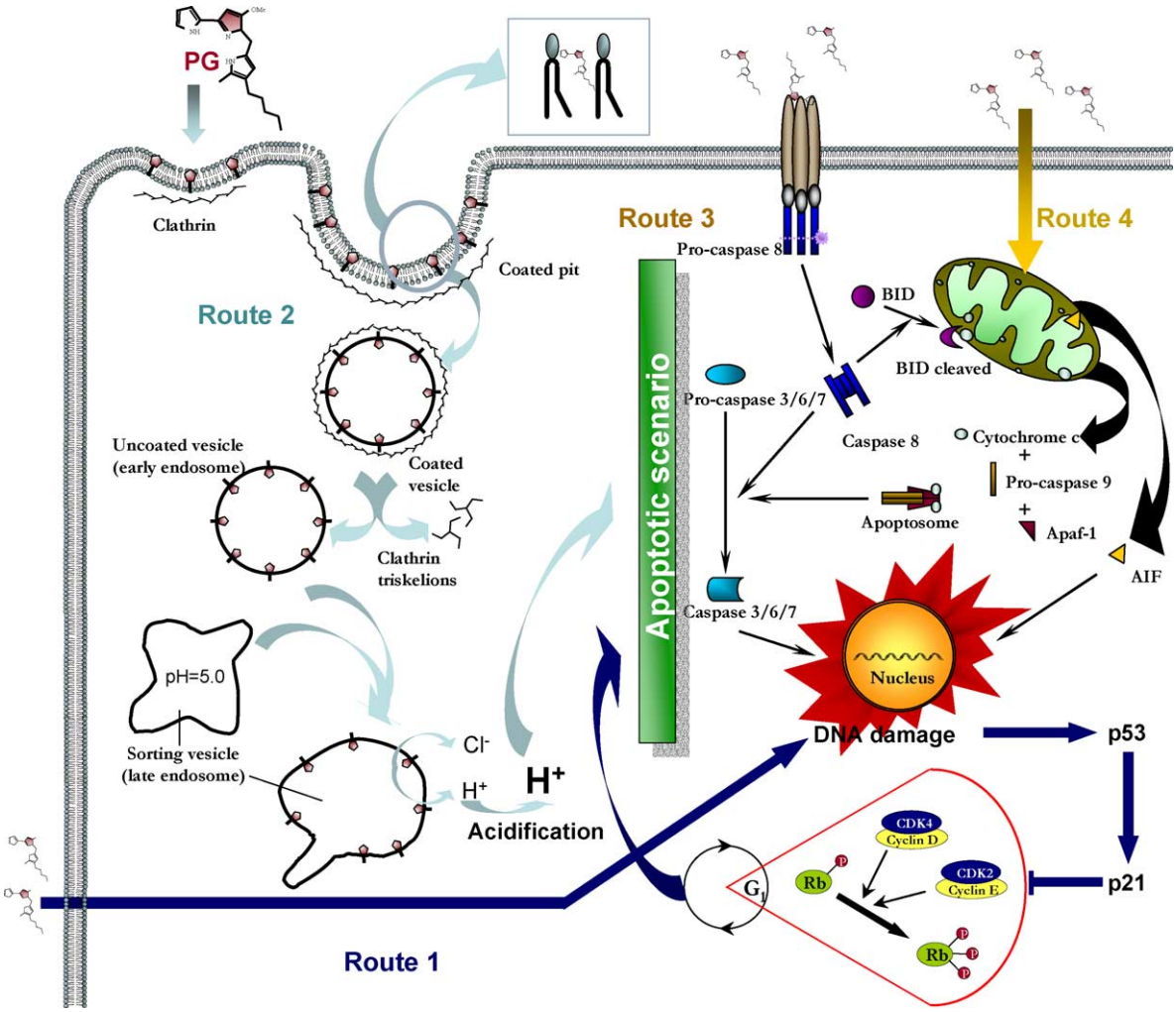

Fig. 2. Scheme for the numerous actions of PG by different pathways. PG could act by simple mechanisms related to its chemical or physical properties. PGs are hydrophobic molecules, very unstable in water solutions, and might diffuse freely through membranes and interact with the DNA with a preference for AT sites from the minor-groove [55] promoting dsDNA cleavage event [59,60]. Cells respond to DNA damage by activating cell cycle arrest [18,22,28,32], DNA repair, and in some circumstances, the triggering of apoptosis (Route 1). PG might be incorporated into the lipids bilayer of the plasmatic membrane, where by endocytosis it reaches the endosome compartment, uncouples vacuolar H^+-ATPase (V-ATPase) through promotion of the H^+/Cl^- symporter, and induces neutralisation of the acid compartment of cells, inducing intracellular acidification and eventually apoptosis (Route 2) [37,38,40,45]. Apoptosis by PG might occur through the activation of an unidentified PG receptor or by the activation of a known death receptor, inducing caspase 8 activation and consequently, apoptosis (Route 3). Finally, PG might diffuse freely through membranes and interact with the mitochondrial outer membrane, uncoupling F_o-F_1-ATPase and therefore, inducing apoptosis (Route 4) [47,48]. In conclusion, the pathway followed by PGs would depend very much on the cell type studied, the drug concentration inside the cell, the hierarchy of the PG targets and the interaction of distinct pathways mentioned above.

membrane transporters could be employed for therapeutic purposes in the modulation of apoptosis pathways *in vivo*.

2.2. PGs as cell cycle inhibitors

Cell cycle-related proteins and cytoplasmic pH homeostasis are connected [49]. In fact, PGs induce cell cycle arrest, although differences exist in the process induced by them, suggesting different mechanisms of action.

UP and PNU156804 induce growth arrest in late G_1 in T and B lymphocytes but not in human Jurkat T [18,22,28]. However, PG inhibits the proliferation of human Jurkat T cells mainly *via* G_1–S transition arrest (Fig. 2, Route 1) [32]. These three molecules abolish the expression of the cyclin-dependent kinase inhibitor p27, suggesting that p27, in their presence, coordinates the final outcome of proliferation or death of the cell [18,22,32]. Furthermore, UP and PNU156804 require the stimulation of Jak3, whereas cPrG·HCl or PG do not need previous stimulation to induce cell cycle arrest in transformed cell lines [18,22,27,28,32,37,44]. cPrG·HCl inhibits proliferation and induces apoptosis in liver carcinoma cell lines [40] and in human breast cancer cell lines [37], and induces differentiation in the human promyelocytic leukaemia cell line HL-60 [38].

Genetic alterations of the p53 tumour suppressor gene are frequently associated with human cancers [50] and give a consistently poor prognosis [51]. The absence of p53 or

aberrant p53 implies that apoptosis does not occur even when the cell suffers genetic damage [52]. PG-induced apoptosis is p53-independent [29], which may represent an advantage over other chemotherapeutic drugs [52,53].

Comparison of the cytotoxic properties of PG (2-methyl-3-pentyl-6-methoxyprodigiosene), prodigiosene and 2-methyl-3-pentylprodigiosene revealed the exceptional cytotoxic potency of PG, which may be attributed to the presence of the PG C-6 methoxy substituent (Fig. 1) [9,29]. Also, differences in the chemical structures of the A-pyrrole rings between PG and UP (2-undecyl-6-methoxyprodigiosene) are key in cytotoxic potency [54]. D'Alessio and co-workers found that the replacement of methoxy by a larger alkoxy steadily reduces activity, which is counterbalanced by a more marked decrease in cytotoxicity, thus favouring selectivity. The best compound with these characteristics obtained by D'Alessio and co-workers was PNU156804, which had a therapeutic index almost 3 times higher than UP [34–36].

2.3. PGs as DNA cleavage agents

DNA-binding molecules regulate mechanisms central to cellular function, including DNA replication and gene expression. The planar PG nucleus binds DNA by intercalation, while the methoxy group and ring nitrogens provide hydrogen-bonding sites to facilitate DNA binding. The cationic nature of PGs at neutral pH also provides electrostatic interaction with the negatively phosphate groups of the DNA helix. PG is a DNA interacting agent, with a preference for AT sites from the minor-groove [55]. In addition, PG facilitates copper-promoted oxidative double strand (ds) DNA cleavage through reductive activation of Cu(II), by oxidation of the electron-rich PG molecule [55,56]. Copper is an essential trace element distributed in all cellular organelles, including nucleus [56,57]. Copper levels are usually high in cancer [58]. In dry non-cancerous breast tissue, the mean concentration of copper is 1.47 ppm, whereas the mean concentration increases to 5.12 ppm in cancerous tissue. Melvin *et al*. predicted that the amount of damage under these conditions would be significant and should be lethal to the cells. They also suggested a correlation between nuclease activity and the cytotoxicity of PG [59].

The A-pyrrole ring of PGs influences the redox properties of pyrromethene. The bipyrrole moiety promotes ssDNA cleavage, while the intact pyrrolylpyrromethene chromophore of PGs is required for the more lethal copper-promoted dsDNA cleavage event [59,60].

Cells respond to DNA damage by activating a complex DNA-damage response pathway that includes cell cycle arrest, DNA repair and, under some circumstances, the triggering of apoptosis (Fig. 2, Route 1) [61,62]. Because PGs bind to DNA, they are capable of disrupting its replication and inducing apoptosis, as we related above, and hence are prospective anticancer drugs.

2.4. PGs as MAPK regulators

Various effects of PGs on MAPK signalling cascades have been described. These cascades include the ERKs, normally associated with proliferation and growth factors, and stress-activated protein kinase (SAPK)/c-*jun* N-terminal kinase (JNK) and p38-MAPK, induced by stress responses and cytokines and a mediator of differentiation and cell death [63].

Protein kinase C (PKC) is involved in many cellular functions, including cell proliferation and differentiation. PKC also participates in the regulation of apoptosis induced by several distinct stimuli, such as TNFα, ionising irradiation and antitumour drugs [64–66]. The activation of PKC by the phorbol ester PMA, which prevents intracellular acidification through PKC-induced activation of the Na^+/H^+ antiport [67], conferred protection against apoptosis induced by PG through an ERK-dependent pathway (Fig. 2, Route 2) [68], whereas the percentage of dead cells increased with cPrG·HCl [19]. The differences in the chemical structures of PG and cPrG·HCl may explain this difference. Moreover, imidazole, a permeable base, prevented intracellular acidification and suppressed cPrG·HCl-induced apoptosis [37].

PGs also activate either or both of the p38-MAPK and SAPK/JNK pathways, so inducing apoptosis. Whereas PG induced phosphorylation of p38 but not of JNK-MAPK [69], cPrG·HCl activated SAPK/JNK to promote apoptosis [38], which suggests that structural or methodological differences account for these discrepancies.

3. Conclusions

The cytotoxic properties of PGs, tripyrrole red pigments, have been recognised for some times. In 1977, Fullan *et al*. observed the antitumour activity of PG in mice [70]. Since then, the results presented above for different cell lines and xenografted nude mice have demonstrated that PG group natural products are promising antineoplastic agents.

Some cancer chemotherapy agents act primarily by causing apoptotic cell death in susceptible cancer cells. Each chemotherapeutic agent interacts with a specific target, causing dysfunction and injury, which is then interpreted by susceptible cancer cells as an instruction to undergo apoptosis [71]. New therapies seek to identify drugs more selectively so as to target more effectively cancer but not normal cells. The identification of novel targets and the development of drugs with greater selectivity towards cancer cells represent the primary goals of cancer therapy research. The *in vitro* 60 human tumour cell panel of the National Cancer Institute Drug Discovery Program (NCI, Bethesda, MD) provides an interesting tool that is available on Internet at www.dtp.nci.nih.gov with the NSC Number: 47147-F. PG has been screened with an average IC_{50} of 2.1 μM.

PGs, therefore, are a new group of molecules with a common mechanism of action to select molecular targets. Although PGs' apoptotic mechanisms are still to be fully determined (additional *in vivo* assays are necessary), current results reported in this review suggest that PGs are a new class of anticancer drugs which hold out considerable promise for the Pharmacological Industry.

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