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Induction of apoptosis by pyrazolo[3,4-*d*]pyridazine derivative in lung cancer cells via disruption of *Bcl-2*/*Bax* expression balance

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ABSTRACT

In the rapidly expanding era of cancer target therapy, regulators of apoptosis are emerging as attractive therapeutic targets. X-linked inhibitor of apoptosis (XIAP) is of specific interest owing to its characteristic overexpression in a wide variety of neoplasms, with a resultant survival advantage for tumor cells and treatment resistance. In this study, we examined three pyrazolo [3,4-*d*] pyridazine derivatives (PPDs) through molecular modeling and studied their modes of interaction with XIAP-BIR3 domain. PPD-1, which possessed the highest binding affinity with XIAP, was tested on A549 (lung cancer cell line); HCT-116 (colorectal carcinoma cell line); HEPG2 (liver carcinoma cell line), HFB4 (normal human skin melanocyte cell line) and WI-38 (human embryonic lung fibroblasts). In comparison to cisplatin as a positive control, PPD-1 yielded remarkable cytotoxicity on all cancer cell lines, with the highest anti-tumor activity on A549 and a favorable therapeutic ratio. Flow cytometry studies concluded that PPD-1 treatment induces Sub G1 and G2/M cell cycle arrest and apoptosis. The percentage of apoptotic cells in PPD-1 treated A549 cells was considerably higher than that in untreated cells (10.06% vs 0.57%, respectively). To further investigate the mechanism of induction of apoptosis by PPD-1, Real time-PCR was used to quantify the expression levels of key apoptotic regulators. Significant overexpression of the effector *capsase-3*, pro-apoptotic *bax* and tumor suppressor gene *p53* were noted as compared to untreated cells (7.19 folds, 7.28 folds, and 5.08 folds, respectively). Moreover, PPD-1 inhibited the expression of the anti-apoptotic *bcl-2* gene to 0.22 folds. These findings demonstrate that PPD-1 treatment disrupts the Bcl-2/BAX balance in lung cancer cell lines, leading to apoptosis induction possibly through intrinsic mitochondria-dependent pathway. These novel insights elucidate the mechanism of PPD-1 cytotoxicity in lung cancer cell lines and offer a promising therapeutic approach that needs further study.

1. Introduction

Among the characteristic features of carcinogenesis are evasion of apoptosis and uncontrolled proliferation of tumor cells.[1] Apoptosis is a strictly regulated process of programmed cell death, which can be triggered by different physiological and pathological stimuli. Several apoptotic pathways have been identified to date, the most significant of which are the intrinsic mitochondria-dependent and the extrinsic death receptor-dependent pathways.[2] This complicated process involves the activation of various initiator and effector caspases in a sequential manner, resulting in morphological cellular changes, including cell shrinkage, condensation of chromatin, and formation of apoptotic bodies.[2] The initiation of apoptosis leads to inactivation of various survival pathways; thus, its evasion critically predisposes to sustained survival, development and proliferation of malignant cells.[1,2]

Inhibitor of apoptosis (IAP) proteins are a family of apoptosis regulators that have been discovered to be over expressed in various solid and hematological malignancies. Their overexpression contributes to sustained proliferation of cancer cells and resistance to chemotherapeutics as well as radiotherapy.[3] X-linked IAP (XIAP) is a well-identified IAP family member that is capable of inhibiting apoptosis, partly by binding caspases-3, -7 and -9.[4] These observations compel the exploitation of targeting IAP proteins in cancer treatment.

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In view of the paramount importance of physiological interactions between proteins and their respective ligands in regulating homeostasis, the development of drugs targeting protein binding is an emerging subject of interest.[5] Most tumor cells possess the ability of inhibiting apoptosis, a feature that provides survival advantage and confers treatment resistance. Recent studies have concluded that targeting apoptosis regulators is a promising anticancer therapeutic approach.[6] Numerous clinical trials are underway investigating the efficacy and safety of pro-apoptotic agents in cancer treatment.[6] Several genes have been identified to regulate apoptosis, of which the tumor suppressor p53 acts as pro-apoptotic gene in response to stimuli inducing DNA damage. Upon activation of p53 protein, it binds to specific target genes promoting cell cycle arrest, DNA repair or apoptosis in case of defective repair.[7] This aims at eliminating cells with damaged DNA and preserving genomic integrity.

Notably, DNA damage also leads to the activation of mitochondria-dependent apoptotic pathway that is mediated by B-cell leukemia/lymphoma-2 (Bcl-2) protein family.[8] This family comprises several pro- and anti-apoptotic proteins which are pivotal in regulating the apoptotic process. The interaction between pro-apoptotic BAK in the mitochondria, and BAX in the cytoplasm, result in mitochondria outer membrane permeabilization and the release of cytochrome *c*.[8] The latter, together with apoptotic protease-activating factor-1 (Apaf-1), act to activate caspase-9, which consequently activates caspases-3, -6 and -7, known as effector caspases.[9,10] The anti-apoptotic Bcl-2 protein serves as an ideal therapeutic target owing to its overexpression in various tumor types.[11]

Lung cancer is one of the most challenging and highly fatal human cancers worldwide. It is the most common cause of cancer death in the United States.[12] XIAP has been repeatedly documented to be overexpressed in lung cancer, leading to apoptosis inhibition and treatment resistance.[13,14] These findings have led to the development of XIAP-targeted therapies that reverse chemo-resistance and offer novel anti-cancer therapeutic approaches.[15]

This study aims at screening novel drugs for their pro-apoptotic activity. Due to the promising biological activities of pyrazole and pyrazolo-pyridazine derivatives as potential agents in targeting cancer,[16] three pyrazolo [3,4-*d*] pyridazine derivatives (PPDs) were designed and their interaction with XIAP-BIR3 domain was investigated. Subsequently, PPD-1 which bound XIAP with the highest affinity was characterized. MTT assay was used for screening of activity against various cancer and normal cell lines, in comparison to cisplatin which is a standard chemotherapeutic agent used in lung cancer treatment. We then selected A549 cells, which showed the highest cytotoxicity, to illustrate whether induction of apoptosis was the underlying mechanism of anti-tumor activity. Cell cycle and apoptosis were investigated using flow cytometry, while the expression levels of apoptotic regulators (*bcl-2*, *bax*, *p53*, and *caspase-3*) were studied using real time polymerase chain reaction.

2. Materials and methods

2.1. Molecular modeling

The three designed PPDs (PPD-1, PPD-2 and PPD-3) were drawn on the docking program. The tested compounds and the used protein model were prepared as in Ali et al[17] and saved for docking. Protein model was obtained from Brookhaven Protein Data Bank (www.RCSB.org). The crystal structure of XIAP-BIR3 in a complex with the Smac protein (PDB code: 1G73) was used to predict the binding models of XIAP-BIR3 bound to the designed PPDs. Docking calculations were carried out using Docking Server.

2.2. Chemicals

RPMI-1640 media, DMEM media, dimethylsulfoxide (DMSO), fetal bovine serum, MTT, RNAse A and propidium iodide (PI) were purchased from Sigma Chemical Co., USA. All other chemicals were of high purity and obtained locally.

2.3. Synthesis of 4-(3-oxo-3H-benzo[f]chromen-2-yl)-2-phenyl-2,6-dihydro-7H-pyrazolo[3,4-d]pyridazin-7-one (4) (PPD-1)

All melting points were measured on a Gallenkamp melting point apparatus (Weiss Gallenkamp, London, UK). The infrared spectra were recorded in potassium bromide disks on a pye Unicam SP 3300 and Shimadzu FT IR 8101 PC infrared spectrophotometers (Pye Unicam Ltd. Cambridge, England and Shimadzu, Tokyo, Japan, respectively). The NMR spectra were recorded on a Varian Mercury VX-300 NMR spectrometer (Varian, Palo Alto, CA, USA). ^{1}H spectra were run at 300 MHz and ^{13}C spectra were run at 75.46 MHz in deuterated chloroform ($CDCl_3$) or dimethyl sulphoxide (DMSO d_6). Chemical shifts were related to that of the solvent. Mass spectra were recorded on a Shimadzu GCMS-QP 1000 EX mass spectrometer (Shimadzu) at 70 eV.

Equimolar amounts of ethyl 2-chloro-2-(2-phenylhydrazono) acetate (**1**), 3-(dimethylamino)prop-2-enoyl]benzo[*f*]2H-chromen-3-one (**2**) and triethylamine (5 mmol) were refluxed in dry toluene (20 mL) for 3 h. The hot solution was filtered off, and the filtrate was evaporated and triturated with petroleum ether (40–60 °C). The resulting solid was collected and crystallized from acetic acid to give ethyl 4-(3-oxo-3*H*-benzo[*f*]chromene-2-carbonyl)-1-phenyl-1*H*-pyrazole-3-carboxylate (**3**). A mixture of the pyrazole **3** (1 g) and hydrazine hydrate (2 mL) in ethanol (20 mL) was refluxed for 2 h. The resulting solid was collected and crystallized to give 4-(3-oxo-3*H*-benzo[*f*]chromen-2-yl)-2-phenyl-2,6-dihydro-7*H*-pyrazolo[3,4-*d*]pyridazin-7-one (**4**) (Scheme 1). Mp. 261–263 °C (AcOH) (Lit mp. 260–263),[18] pale yellow, yield 82%. IR: 3385 (NH), 1705, 1670 (CO's) and 1606 (C=C). ^{1}H NMR: 7.3–8.2 (m, 12H, ArH's), 8.4 (s, 1H, pyrazole H-5), (s, 11.8, NH), ^{13}C NMR: 112.0, 118.9, 119.5, 122.5, 123.4, 125.6, 126.1, 127.2, 127.8, 128.2, 128.5, 129.7, 130.1, 131.3, 133.4, 139.8, 141.2, 143.9, 146.2, 149.8, 155.3, 157.9. Anal Calcd: $C_{24}H_{14}N_4O_3$ C: 70.93, H: 3.47, N: 13.79 Found: 406.396 71.10 3.60 13.60.

2.4. Cell lines and culture conditions

A549 (Lung carcinoma cell line); HCT-116 (Colorectal carcinoma cell line); HEPG2 (liver carcinoma cell line); HFB4 (normal human skin melanocytes cell line) and WI-38 (human embryonic lung fibroblasts) were purchased from American Type Culture Collection and were maintained in their suitable culture media supplemented with 10% fetal bovine serum (Gibco), penicillin/streptomycin (Gibco). All incubations were done at 37 °C in a humidified atmosphere of 5% CO_2. Mycoplasma was tested at 3 months intervals.

2.5. Cell proliferation assay by MTT

Cytotoxicity of the newly synthesized PPD-1 was evaluated on A549 (Lung carcinoma cell line); HCT-116 (Colorectal carcinoma cell line); HEPG2 (liver carcinoma cell line); HFB4 (normal human skin melanocytes cell line) and WI-38 (human embryonic lung fibroblasts) 48 h post treatment using MTT assay. In order to investigate the potential of PPD-1, cisplatin -a commercial classical anticancer drug- was used as a reference. PPD-1 and cisplatin were dissolved in DMSO to prepare stock concentrations of 5 mg/mL. The stock solutions were filtered through 0.22 μm syringe filter. Double fold dilutions were prepared by adding equal volumes of