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Development and validation of a novel assay to identify radiosensitizers that target nucleophosmin 1

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ABSTRACT

A series of indole analogs that are synthesized using the scaffold of a potent radiosensitizer, YTR107, were tested for their ability to alter the solubility of phosphorylated nucleophosmin 1 (pNPM1). NPM1 is critical for DNA double strand break (DSB) repair. In response to formation of DNA DSBs, phosphorylated T199 NPM1 binds to ubiquitinated chromatin, in a RNF8/RNF168-dependent manner, forming irradiation-induced foci (IRIF) that promote repair of DNA DSBs. A Western blot assay was developed using lead molecule, YTR107, for the purpose of screening newly synthesized molecules that target pNPM1 in irradiated cells. A colony formation assay was used to demonstrate the radiosensitization properties of the compounds. Compounds that enhanced the extractability of pNPM1 upon radiation treatment possessed radiosensitization properties.

1. Introduction

Precision medicines that target specific driver mutations have revolutionized cancer therapy. Unfortunately, not all patients will be able to take advantage of these therapies. Many will present with tumors that do not express actionable molecular driver mutations. Non-small cell lung cancer (NSCLC) represents an excellent example, as 36% of patients fall into this category.[1] Cytotoxic therapy continues to be a very important tool for the treatment of human cancers that do not express actionable molecular targets. Ionizing radiation is a cytotoxic agent that has a central role in cancer therapy and is used to provide local/regional control of cancer;[1] a requirement for preventing tumor-mediated organ failure, tumor recurrence and metastasis.[2–4] Recent advances in 3-D image-guided radiation therapy have significantly increased the probability of obtaining outstanding local/regional tumor control. However, a limitation to this therapy is the intrinsic radiation resistance of individual tumor cells[5] due to increased DNA repair potential.[6–8] Thus, targeting DNA repair represents a rational strategy for overcoming radiation resistance.

The indole structure of the radiation sensitizer, indomethacin[9], was used as a scaffold for the synthesis of a series of (*Z*)-5-((*N*-benzyl-1*H*-indol-3-yl)methylene)imidazolidine-2,4-dione and (*Z*)-5-((*N*-benzyl-1*H*-indol-3-yl)methylene)pyrimidine-2,4,6(*1H,3H,5H*)-trione derivatives that incorporated a variety of aromatic substituents in both the indole and *N*-benzyl moieties. Functional phenotypic screening for structure activity relationships revealed that introduction of the electron withdrawing group 4-CN into the *N*-benzyl moiety yielded a potent radiosensitizing compound[10], capable of sensitizing six NSCLC cell lines, HT29 colorectal cells, D54 glioblastoma cells, PANC1 pancreatic cancer cells, and two breast cancer cell lines.[11,12] This molecule, a substituted (*Z*)-5-((*N*-benzyl-1*H*-indol-3-yl)methylene)pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione was renamed YTR107 (Fig. 1).

Inhibition of DNA double strand break (DSB) repair represented the mechanism responsible for YTR107-mediated radiosensitization.[12] YTR107 exhibited efficacious radiosensitization in 2 tumor xenografts and a syngeneic tumor model but did not produce overt normal tissue toxicity[11] or normal tissue radiosensitization (unpublished results).

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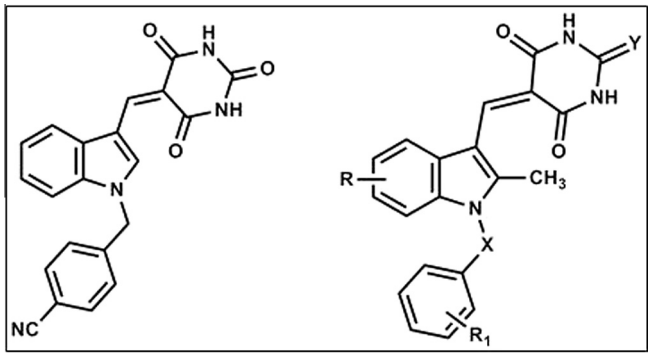


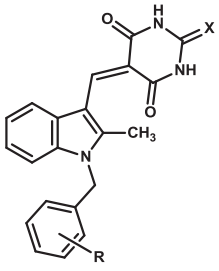
Figure 1. Structures of YTR107 and its scaffold.

Use of YTR107 as a chemical probe resulted in the identification of the non-enzymatic chaperone, nucleophosmin 1 (NPM1) as a biological target, that is, critical for DNA DSB repair. In response to formation of DNA DSBs, phosphorylated T199 NPM1 binds to ubiquitinated chromatin, in a RNF8/RNF168-dependent manner, forming irradiation-induced foci (IRIF) that promote repair of DNA DSBs.[13] YTR107 binds to the amino terminus of NPM1, inhibiting IRIF formation, which in turn impairs DSB repair, and thus acts as a radiosensitizer. Genetic and cell biological approaches validated this concept by demonstrating that NPM1-deficient cells have impaired DNA DSB repair and consequently are radiosensitive. Use of NPM1-null mouse embryo fibroblasts demonstrated that the molecular basis of YTR107-mediated radiosensitization is YTR107 targeting of NPM1 and subsequent inhibition of DNA DSB repair. Although development of the YTR107 probe and discovery of its action represent a critical step for understanding a novel radiosensitizing mechanism, its use is hampered by limitations such as poor water-solubility.

In the present work, we report on a series of novel 2-methyl-*N*-benzyl aplysinopsin analogs, that is, 2-methyl-5-((1-benzyl-1*H*-indol-3-yl)methylene)-2-oxodihydropyrimidine-4,6(1*H*,5*H*)-triones and 2-methyl-5-((1-benzyl-1*H*-indol-3-yl)methylene)-2-thioxodihydro-pyrimidine-4,6(1*H*,5*H*)diones (Fig. 2), which have been evaluated in a novel screening assay for their ability to modulate the extractability of phospho-nucleophosmin 1 (pNPM1 or pT199NPM1) after radiation treatment. The synthesis and anticancer properties of these analogues have recently been reported by us.[14] The screening assay was developed based on our novel observation that exposure of irradiated cancer cells to YTR107 increases the extractability of nuclear pNPM1 in high salt extraction buffer when compared to solvent control.[11] In the present report, we analyzed the solubility of pNPM1 in NP-40 and RIPA buffers and correlated the findings with radiosensitization of H460 lung cancer cells with these novel YTR107 analogs.

1.1. Methods and buffer compositions

Lung cancer cells, A549, H460, and Calu1 as well as normal human lung fibroblasts, IMR-90 cells were purchased from ATCC and cultured in DMEM/F-12 (A549), RPMI-1640 (H460), and



1a-r

Compound	ID	R	X
1a	PNR-5-81	H	O
1b	PNR-5-82	H	S
1c	PNR-5-84	4-CN	O
1d	PNR-5-85	4-CN	S
1e	PNR-5-87	4-COOCH3	O
1f	PNR-5-88	4-COOCH3	S
1g	PNR-5-90	3,4,5-trimethoxy	O
1h	PNR-5-91	3,4,5-trimethoxy	S
1i	PNR-5-95	3,5-dimethoxy	O
1j	PNR-5-96	3,5-dimethoxy	S
1k	PNR-6-01	2-Br	O
1l	PNR-6-02	2-Br	S
1m	PNR-6-04	4-Br	O
1n	PNR-6-05	4-Br	S
1o	PNR-6-07	2,4-dimethoxy	O
1p	PNR-6-08	2,4-dimethoxy	S
1q	PNR-6-10	2-CF_3	O
1r	PNR-6-11	2-CF_3	S

Figure 2. Structures of 2-methyl-5-((1-benzyl-1*H*-indol-3-yl)methylene)-2-oxodihydropyrimidine-4,6(1*H*,5*H*)-triones and 2-methyl-5-((1-benzyl-1*H*-indol-3-yl)methylene)-2-thioxodihydropyrimidine-4,6(1*H*,5*H*)diones (**1a**–**r**).