Graphical Abstract

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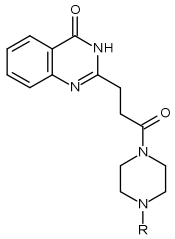

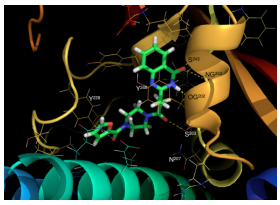

Bioorganic & Medicinal Chemistry Letters
journal homepage: www.elsevier.com

Novel PARP-1 inhibitors based on a 3H-quinazolin-4-one scaffold

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ARTICLE INFO

Article history:
Received
Revised
Accepted
Available online

Keywords:
PARP-1inhibitors Anticancer
3-(4-Oxo-3,4-dihydro-quinazolin-2-yl)-propionamidesMolecular Modeling
Virtual screening of commercial libraries
Hit-to-lead optimization

ABSTRACT

Poly(ADP-ribose)polymerase-I (PARP-1) enzyme is involved in maintaining DNA integrity and programmed cell death. A virtual screening of commercial libraries led to the identification of five novel inhibitors having 4-(methylene)-4H-isoquinolin-1,3-dione, 1(2H)-phthalazinone, 2,4-(1H,3H)-quinazolinedione, 4(3H)-quinazolinone and 2-oxomethylenebenzamide scaffolds with low nanomolar IC_{50} values (28.5-87.6 nM). Hit-to-lead optimization, undertaken on the most active compound of the series, let to identification of a group of new potent PARP-1 inhibitors, acylpiperazinylamides of 3-(4-oxo-3,4-dihydro-quinazolin-2-yl)-propionic acidMolecular modeling studies highlighted the importance of the propanoyl side chain for the activity.

Poly (ADP-ribose) polymerases (PARPs) are a family of proteins whose main roles involve maintaining DNA integrity and programmed cell death. So far 18 members have been recognized, but PARP-1 and PARP-2 are the two most studied isoforms. PARP-mediated DNA repair utilizes base excision repair pathway.[1] Blocking PARP's activity prevents DNA damage repair, which finally leads to cell death through induction of DNA double-strand breaks (DSBs). Lately, however, new mechanisms of action of PARP inhibitors have been discovered. It has been demonstrated that PARP protein, when bound to some inhibitors, instead of being released from DNA once the repair process has started, remains trapped on DNA preventing its replication and consequently cell division.[2]

PARP-mediated repair process involves the binding of damaged DNA to N-terminal zinc finger motif of PARP, which in turn causes activation of the catalytic C-terminal domain and

allows the enzyme to hydrolyze NAD^+, , ultimately resulting in the production of linear and branched poly-ADP-ribose chains.

Enhanced PARP-1 expression, and/or activity, has been also observed in different human tumor cell lines such as hepatocellular carcinoma, colorectal carcinoma, cervical carcinoma, malignant lymphomas, and leukaemia. Moreover, it has been reported that PARP catalytic activity is also stimulated in response to DNA damage.[4]

It is well-known that many anti-cancer therapies, such as those based on temozolomide (TMZ), platinum-based drugs, topoisomerase inhibitors and radiotherapy, implicate DNA damage. However, these therapies are shadowed by the emergence of resistance, notably due to DNA repair through PARP pathway, undermining their efficacy. Such observation led to the development of combination therapies wherein resistance to the mechanism of action of the DNA damaging drugs was hampered by PARP inhibition. In fact, it has even been shown that PARP inhibition could potentiate the effect of DNA damaging agents[5,6], as well as radiotherapy. Cancer cells presenting at least one of BRCA1 and BRCA2 mutated genes, two well-known tumor-suppressor genes, are very sensitive to PARP-1 inhibition, resulting in cell cycle arrest and apoptosis. This suggests an efficacious role for PARP inhibitors, as single agents, against tumors exhibiting BRCA1 and/or BRCA2 mutations.[7-9]

As a result of all these discoveries, a series of PARP-1 inhibitors has been successfully produced and assessed in advanced clinical trials, either as stand-alone monotherapies or as combination therapies. All these compounds have a nicotinamide-based structure aimed to compete with NAD^+ for the binding to PARP-1 catalytic site [e.g., olaparib (AZD2281), veliparib (ABT-888), niraparib (MK-4827), BMN-673, and rucaparib (AG-014699/ PF-01367338)] (Figure 1).

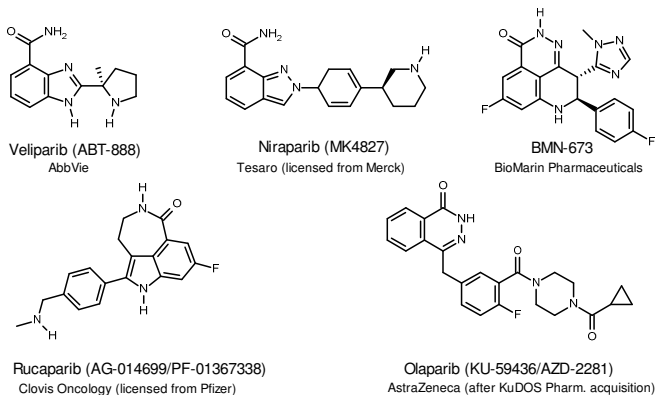


Figure1: Structure of some PARP inhibitors

X-Ray crystal structures[11,12] and molecular modelling studies[13-16] have indicated that the amide of nicotinamide makes three key hydrogen bonds with the hydroxyl group of S904 and the amide backbone of G863, and that there is a stacking interaction with a conserved Y907. The synthesis of conformationally constrained cyclic derivatives has allowed the demonstration of an anti-disposition of the amide bond. Thus, attempts to improve the affinity of PARP inhibitors to the binding site have been made by trying to lock the carboxamide group, which is usually free to rotate. This locking is made possible either by inserting on the aromatic ring heteroatoms or groups able to give an intramolecular hydrogen bond with the amide NH, or by enclosing the amide group into a two (or more)-ring heterocycle.[11]

Based on these findings, we created a pharmacophore query in which three main structural features were taken into account: 1) an aromatic ring, 2) a carboxamide moiety with at least one NH group locked into the desired anti-conformation either by enclosure into a ring system or by the formation of intramolecular hydrogen bonds, 3) a side chain extending into the deep pocket located in the auto-modification domain of PARP-1, linked to the 2 or 3 position relative to the carboxamide group (Figure 2).

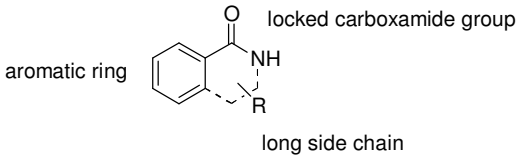


Figure 2. Pharmacophore query

A virtual screening of commercial libraries led to the identification of a few scaffolds containing appropriate chains and/or groups that satisfied the pharmacophore query, i.e. 4-(methylene)-4H-isoquinoline-1,3-dione (group A), 3H-quinazoline-4-one, (B) 1H-quinazoline-2,4-dione (C), 2H-phthalazine-1-one (D) and 2-oxomethylenebenzamide (E) derivatives (Figure 3).

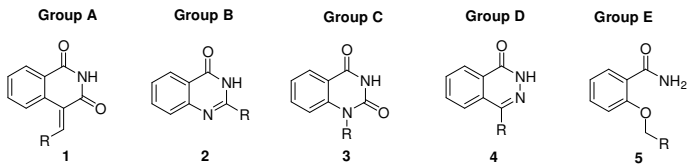


Figure 3. Scaffolds selected by a virtual screening of commercial libraries.

Three derivatives from group A, seven from each of groups B and C, nine from group D, and six from group E were evaluated for their inhibitory activity against recombinant human PARP-1, expressed as GST fusion protein, by means of a highly sensitive fluorescent enzymatic assay (see Supporting Information).

Moreover, based on the hypothesis that PARP-1 inhibitors could represent a monotherapy option against tumors with mutated BRCA1/2 genes, as also suggested by several experiments performed in vitro and in vivo on human tumor cell lines or xenograft models, respectively[17,18], ~~For this reason,~~ The most active compounds of each group (Figure 4) were subjected to further investigation, i.e. cytotoxicity against the triple-negative and BRCA-1 deficient breast tumor cell line MDA-MB436, ~~a breast cancer. mi sembra una frase troppo lunga c~~

All tested compounds appeared to be potent inhibitors of PARP-1 enzymatic activity, in the nM range (Figure 4), but had a weak antiproliferative activity on the above mentioned breast cancer cell line (> 10 μM).

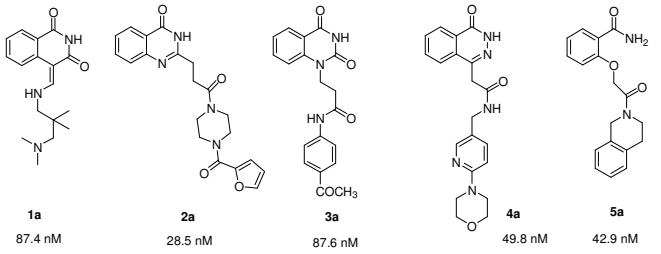


Figure 4. Structure and PARP-1 inhibitory activity (IC_{50}) of the selected compounds.

The most active compound of the series, **2a**, appeared as a promising hit, so that optimizing modifications were undertaken.

Although a number of quinazolinone derivatives with PARP inhibiting activity have been already reported,[x-19-22] none of them contains a propanoyl chain attached to the position 2 of the quinazolinone nucleus. We thought that the CO group of this chain could have been an important point of interaction with a suitable hydrogen bond donating group in the receptor.

This hypothesis was confirmed by docking some compounds in the PARP-1[11] protein and optimizing the interactions by a QM/MM mixed approach. All of the tested compounds behave as inhibitors on the PARP-1 protein, anchoring to the nicotinamide binding site in a very similar way. The compounds are placed inside the cavity, with the main heterocyclic ring inserted between Y246 and H201, forming a strong pi-pi interaction with Y246. The ring carboxamide group gives rise to ~~threeone~~ hydrogen bond with the S243 OH group, and two with the G202 backbone. This situation is represented in Figure 5 for the hit compound **2a**.

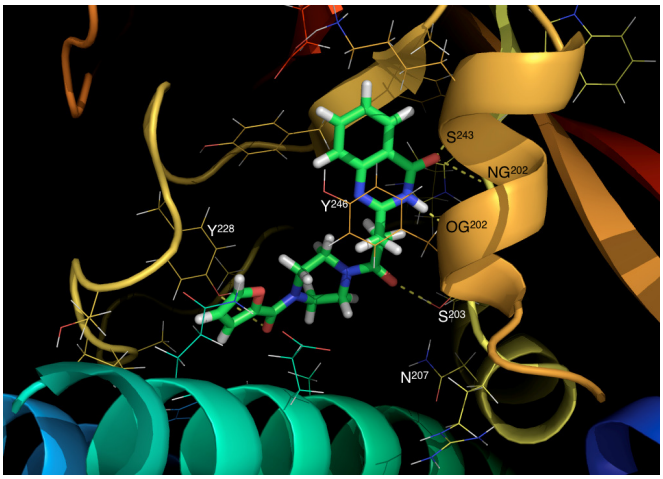


Figure 5. Representation of **2a** binding mode with the key residues labelled

The presence of one or more carbonyl groups in the side chain was found to be an important point of interaction with the receptor, ~~through~~ allowing to ~~the~~ formation ~~of~~ hydrogen bonds with various residues of PARP-1. Considering again **2a**, the carbonyl group of the propanoyl chain forms a very strong hydrogen bond with the S203 hydroxyl group (1.70 Å). The pattern of interaction with PARP-1 is completed by a hydrogen bond between the second carbonyl group and the hydroxyl group of Y228, with a distance of 1.80Å. In this orientation, the furan moiety is placed in front of the aromatic ring of Y228, thus being able to form a strong pi-pi interaction (Figure 5).

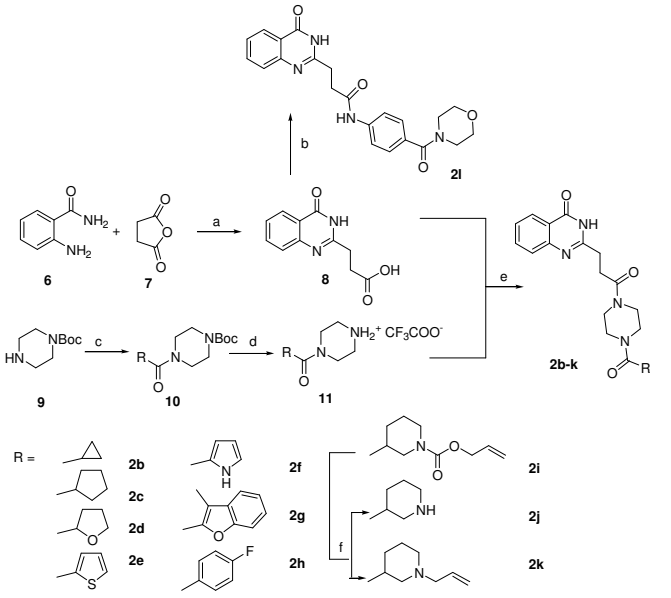
As a matter of fact, most recent reports appeared when this work was already completed, indicating a similar interaction of this carbonyl group with N387 (through a water molecule) of PARP-3[23] and with Y1213 of Tankyrase-1.[24]

The importance of the carbonyl group for the activity of the herein described compounds was confirmed by studying the derivatives lacking the carbonyl. Indeed, the replacement of the carbonyl group with a methylene group preclude the compounds to interact effectively with the receptor, leading to a 10- to 100-fold reduction of the calculated free energy. The observed effect is mainly due to the inability of the methylene group to get close to region delimited by S203 and N207. The different orientation within the binding site leads to the lack of the interactions with S203 and Y228, resulting in a dramatic fall of the affinity for the PARP-1 receptor. (Fig.. in SI)

Hit-to-lead optimization was performed by keeping intact the nucleus with the propanoyl-piperazino chain (**2a**), and modifying the acyl moiety (2-furanoyl in the case of **2a**), introducing cycloalkyl or aromatic groups (**2b-c, 2h**) or heterocycles (tetrahydrofuran, thiophene, pyrrole, benzofuran, piperidine) (**2d-g, 2l-k**) (Scheme 1).

The key compound for the synthesis of the series of compounds **2** was quinazolin-4(3H)-one-2-propanoic acid **8**, in turn prepared from anthranilamide and succinic anhydride by heating in toluene[25], followed by ring closure with NaOH.

The acylpiperazines were obtained by coupling commercial N-Boc-protected piperazine and the appropriate acid by standard peptide chemistry (PyBOP, DIPEA, DMF, rt)[26], followed by deprotection with TFA/CH_2Cl_2 to give the corresponding trifluoroacetates **11**. These were coupled with acid **8** by peptide coupling chemistry too. Compound **2k** was obtained from **2i** by treatment with $Pd(PPh_3)_4$ and morpholine as a side product of **2j**.



Scheme 1. Reagents and conditions: (a) i. Toluene, reflux; ii. NaOH 2N, reflux (83%); (b) (4-aminophenyl)-4-morpholinylmethanone, TEA, HATU, pyridine, 140 °C, MW (**2l**: 60%); (c) RCOOH, PyBOP, DIPEA, DMF, rt (47-80%); (d) DCM/TFA=7/3, rt, quantitative; (e) PyBOP, DIPEA, DMF, rt (**2b**: 50%; **2c**: 73%; **2d**: 48%; **2e**: 38%; **2f**: 51%; **2g**: 62%; **2h**: 52%; **2i**: 32%); (f) $Pd(PPh_3)_4$, morpholine, DCM, rt (**2j**: 36%, **2k**: 17%).

Among these new derivatives, **2e** was shown to inhibit PARP-1 catalytic activity with potency 3- fold higher than that of parental **2a** (Table 1), whereas other compounds showed a comparable (**2f** and **2j**) or a slightly weaker (**2b** and **2h**) inhibitory activity with respect to **2a.** This is consistent with the presence of a ring, in these compounds, which replaces the furan ring maintaining the pi-pi interaction with Y228, as shown by the modelling.

A moderate reduction of inhibitory activity was instead observed for derivatives **2c**, **2d** and **2g**, whereas more pronounced was the decrease in activity for **2k** (IC_{50}=220 nM) and **2i** (IC_{50}=300 nM).

Some of the most active compounds (**2b, 2e, 2f**, and **2h**) were then assessed through a functional parylation assay on a cellular model (human endometrial carcinoma cell line HeLa), which allows to obtain information on the ability of tested compounds to affect the PARP-mediated parylation of nuclear proteins following a strong DNA damage induced by treatment with H_2O_2.[27]

As shown in Table 2, all the compounds showed a relevant inhibitory activity also on this cellular model, with EC_{50} values ranging from 256 nM (**2e**) to 865 nM (**2f**), thus confirming their ability to target and inhibit PARP-1 activity.

Table 1. PARP-1 inhibiting activity of compounds **2**

Cpd.	PARP-1; IC_{50} (nM)±SD[a]
2a	28.5±0.8
2b	67.1±4.6
2c	110.0±70.0
2d	130.0±20.0
2e	9.8±2.5
2f	29.5±3.7
2g	130.0±20.0
2h	56.1±4.7
2i	300.0±80.0
2j	31.9±8.4
2k	220.0±40.0
Olaparib	10±0.2
Veliparib	10±0.2
MK-4827	30±5

[a] Values are the mean (± S.D.) of different experiments (n=3).

Table 2. Results of a PARylation assay on HeLa (human endometrial ca.) cells.[a]

Compound	PARylation; EC_{50} (nM)
2b	707
2e	256
2f	865

2h	467	

[a] Values are the mean of two experiments (standard deviations were within 25% of the mean values).

Although temozolomide (TMZ), topoisomerase I poisons and ionizing radiation (IR), as mentioned above, associated with a PARP inhibitor increase the anticancer activity in a wide range of tumor models, the most important aspect is the discovery that these inhibitors alone selectively kill cancer cells that lack homologous recombination (HR) without affecting repair competent cells. This observation has rapidly translated into clinical trials where PARP inhibitor have shown good anticancer activity in BRAC1 and BRAC2 patients with breast, ovarian and prostate cancer.

For this reason, compounds **2b, 2e, 2f, and 2h** were also investigated alone and in combination with TMZ on triple-negative, BRCA1-mutated, MDA-MB436 breast carcinoma cells and the combination index indicated a synergic effect (Table 3).

Table 3. Combination Index (CI) values of interactions between selected test compounds and temozolomide (TMZ), calculated by CalcuSyn method, on MDA-MB436 breast triple-negative, BRCA1-mutated, cells after 7 days of treatment.

Cells	**Compound**	**IC_{50}, µM**	**Combination index values (CI)**		
			ED_{50}	ED_{75}	ED_{90}
MDA-MB436	**2b**	51.1	0.70	0.80	0.90
	2e	17.3	0.05	0.22	0.85
	2f	18.1	0.12	0.43	0.80
	2h	19.5	0.40	0.60	0.90
	TMZ	120			

CIs < 1 indicate synergism. Combination index were calculated according to Chou-Talalay method using CalsuSyn software (Biosoft). The drugs were applied at a fixed ratio of the IC_{50} across a range of activities and viability was evaluated using a SRB assay at each dosage.

Compounds **2e** and **2h** were successively selected for a pre-ADME evaluation. In a permeability assay on Caco-2 cells monolayers in which transport of the compounds was studied in both directions (absorption and efflux); , an efflux ratio of 5.8 and 6.0, respectively (moderate permeability) was measured. Evaluation of blood/plasma distribution gave, after 60 minutes, a ratio of about 0.4 for both, whereas human plasma stability studies gave, after 120 minutes, a recovery of 50% for **2e** and 57% for **2h.** Finally, evaluation of metabolic stability on human hepatocytes gave a $t_{1/2}$ of 584 min (**2e**) and 618 min (**2h**). Besides, the two compounds tested were not found to be a substrate of P-gp.

Compound **2e** was selected for an in vivo also investigated in vivo investigation against the MX1 breast carcinoma cells, characterized by BRCA1 deletion and BRCA2 gene mutation, ,xenografted in athymic SCID beige mice. As reported in Table 4, the molecule delivered intraperitoneally at the maximum tolerated dose of 200mg/10mL/kg, according to the schedule qdx5/wx3w , was shown to significantly inhibit (by 47%) the tumor growth ($P<0.01$ *vs*. vehicle treated group, Mann-Whitney) .

In conclusion, a ligand-based approach to discover novel PARP-1 inhibitors was planned, taking into account the pharmacophoric features of known inhibitors.

Five thousand compounds, selected from commercial libraries, were virtually screened against PARP-1, and five new scaffolds were discovered to be active, with IC_{50} values in the low nanomolar range (IC_{50} values 28.5-87.6 nM), against recombinant human PARP-1.

A 4(3H)-quinazolinone hit (**2a**) was selected. Synthetic efforts were made to improve the inhibitory potency against the enzyme. A series of derivatives substituted with different acyl groups was prepared, most of them being active on PARP-1 in a two-digit nanomolar range. The activity of the most potent derivatives was further confirmed in a functional cell parylation assays. The same compounds showed a synergic effect in combinations with temozolomide in vivo. Moreover, when investigated in vivo, compound **2e** revealed to be efficacious in inhibiting the tumor volume of a BRCA1-deleted and BRCA2 mutated breast carcinoma. Because PARP-1 is highly expressed in a variety of cancers, including breast, hepatocellular carcinoma and non-small cell lung cancer, [28,29,30], and its expression is often correlated with poor prognosis and drug-resistance, the identification of novel PARP inhibitors may be worthy of interest.

Taken together, these results demonstrate that the 2-propanoyl-quinazolinone nucleus is a suitable scaffold for the development of new PARP inhibitors. The profile of the selected compounds can be a starting point for a lead optimization.

Table 4. Antitumor activity of **2e** against MX1 human breast carcinoma xenograft

Cpd.	Dose/route mg/kg	Schedule	BWL%	Lethality	TV± (d +38)	TVI% (d +38)
Vehicle	0	Qdx5/wx3w	0	0/8	781±30	/
2e	200/ip	Qdx5/wx3w	10	0/8	412±56	*47

Treatments started 7 days after tumor injection.

*P<0.01 vs vehicle-treated group (Mann-Whitney's test).

Acknowledgment.

The authors would like to thank Prof. Lucio Merlini for helpful suggestions and for assistance with the manuscript.

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Supplementary Material

Supplementary data (1H and MDS data of the synthesized compounds and biological assays) associated with this article can be found, in the online version, at …..