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Novel quinazolin-4-one derivatives as potentiating agents of doxorubicin cytotoxicity

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ABSTRACT

We report the design, synthesis and biological evaluation of 17 novel 8-aryl-2-morpholino-3,4-dihydroquinazoline derivatives based on the standard model of DNA-PK and PI3K inhibitors. Novel compounds are sub-divided into two series where the second series of five derivatives was designed to have a better solubility profile over the first one. A combination of *in vitro* and *in silico* techniques suggested a plausible synergistic effect with doxorubicin of the most potent compound **14d** on cell proliferation via DNA-PK and poly(ADP-ribose) polymerase-1 (PARP-1) inhibition, while alone having a negligible effect on cell proliferation.

1. Introduction

DNA-dependent protein kinase (DNA-PK) is a member of the phosphatidylinositol 3-kinase (PI3K) related protein kinase family (PIKK). With two other members of this group, ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR), they take care of DNA maintenance and manage DNA damage response (DDR) [1]. DNA-PK is the major regulator of the cellular answers to DNA double strand breaks (DSBs), where both strands of the DNA helix are severed. DSBs, often caused by ionizing radiation (IR) or chemotherapeutic drugs, are the most lethal type of DNA lesion and if unrepaired, they can lead to cell death or to genome rearrangements with subsequent malignant transformation. DNA-PK helps the cells to deal with DSBs through activation of non-homologous end joining (NHEJ) [2–4]. NHEJ is a type of DSB repair which does not require a homologue template; it mediates direct ligation of the broken strands, and it is therefore not limited by the cell cycle.

DDR is a highly studied area of current cancer research. Modulation of these complex signaling pathways brings promise as radio- or chemopotentiating agents (IR, chemotherapeutic drugs) and for selective killing of cancer cells through 'synthetic lethality' [5,6]. Inhibitors of PIKK are still at an early stage of their development; recently two ATR inhibitors (VX-970, AZD6738) and two DNA-PK inhibitors (CC-122 and MSC2490484A) have entered into phase I clinical trials [7–10]. A significant element of the DNA-PK and PI3K inhibitors originates from the chromone compound LY294002 (**1**; Fig. 1) [11]. This non-selective inhibitor of PI3K and PIKK members served as a versatile template in the development of a range of selective and potent inhibitors. Basic modifications aimed at increasing the selectivity towards DNA-PK were carried out at the 8-position of the basic chromone scaffold and included aryl/heteroaryl substitution. Introduction of bulky non-polar aromatic substituents in NU7441 (**2**) and KU-0060648 (**3**) was associated with a remarkable increase in DNA-PK inhibition potency

Abbreviations: ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and Rad3-related; DCM, dichloromethane; DDR, DNA-damage response; DNA-PK, DNA-dependent protein kinase; DOX, doxorubicin; DSB, double strand break; GP, growth percentage; IR, ionizing radiation; NHEJ, non-homologous end joining; MTD, maximum tolerated dose; PARP-1, poly(ADP-ribose) polymerase-1; PI3K, phosphatidylinositol 3-kinase; PIKK, phosphatidylinositol 3-kinase related protein kinase; SSB, single-strand break; THF, tetrahydrofuran; Xphos, 2-dicyclohexylphosphino-2′,4′,6′-triisopropylbiphenyl

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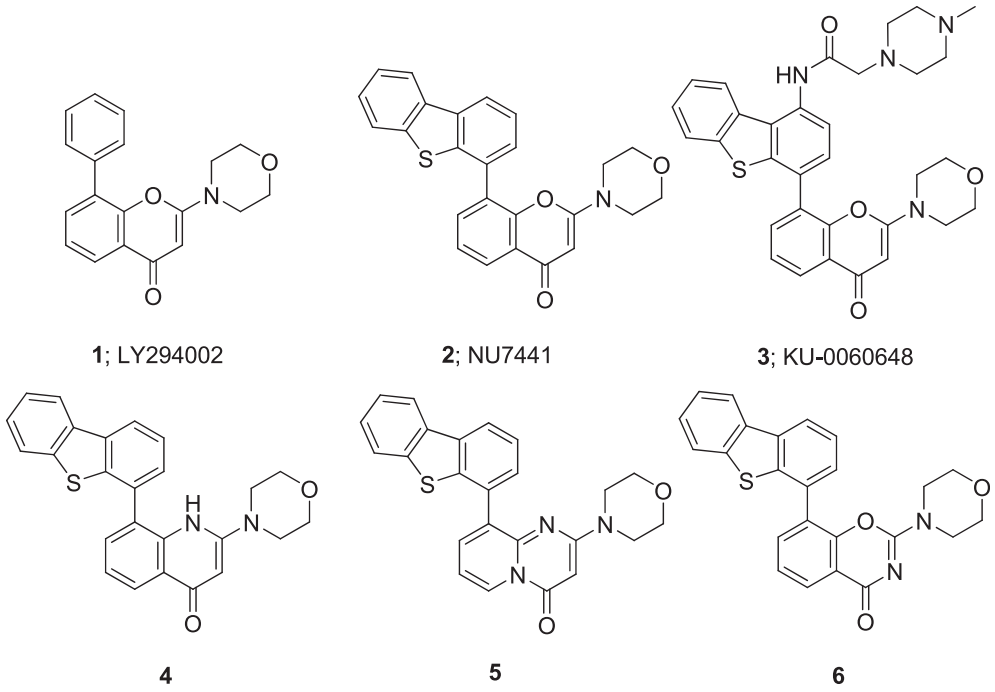


Fig. 1. Structures of known DNA-PK inhibitors.

[12,13]. Core scaffold modification was another extensively pursued possibility to obtain novel lead compounds. Accordingly, quinolinones (**4**), pyrido[1,2–*a*]pyrimidinones (**5**) and 1,3-benzoxazinones (**6**) have been reported. These isosteric compounds proved that the different electronic structures retain their binding activity but display modified selectivity profiles [14–17].

Against this background we discovered a novel series of 3,4-dihydroquinazolinones (**10a-k** and **14a-e**) which could exhibit a new selectivity pattern and activity profile for DNA-PK and PI3K. We present two 2-morpholin-4-yl-3,4-dihydroquinazolin-4-one families, one with a modified aryl moiety at the 8-position of the core heterocycle. A second series was developed through the introduction of polar heads on the attached phenyl to avoid solubility issues which were limiting further biological evaluation [13]. Morpholine represents an essential building block to preserve affinity for DNA-PK and PI3K [14]. *N*-{4-[2-(Morpholin-4-yl)-4-oxo-3,4-dihydroquinazoline-8-yl]phenyl}-2-(piperidin-1-yl)acetamide (**14d**), a compound from the second subset, showed a potent synergic effect with doxorubicin (DOX) in colorectal carcinoma cells HT-29. *In vivo* experiments were carried out in order to assess the maximum tolerated dose (MTD) as well as to reveal the therapeutic relevance of **14d** in female mice. In this article, we present the basic characteristics for the two novel series building on 2-morpholin-4-yl-3,4-dihydroquinazolin-4-one with potential applicability in cancer treatment.

2. Results and discussion

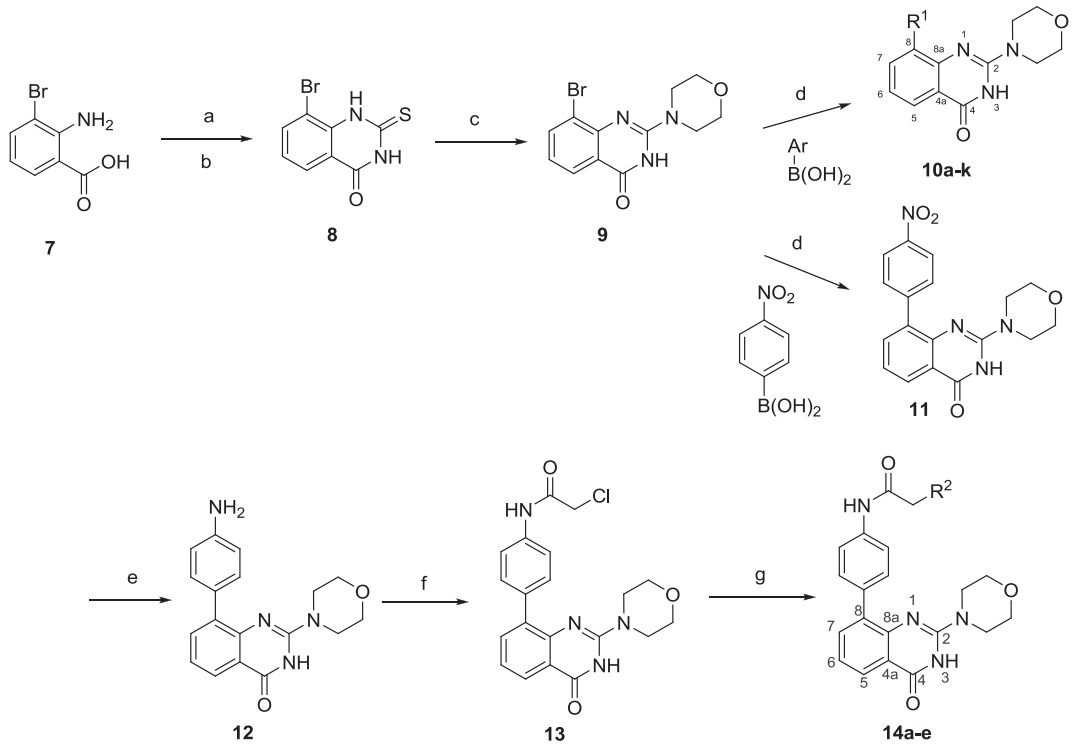
2.1. Chemistry

The compounds were synthesized as outlined in Scheme 1. Chlorination of 2-amino-3-bromobenzoic acid (**7**) with thionyl chloride and subsequent reaction with ammonium thiocyanate gave 8-bromo-2--sulfanyliden-1,2,3,4-tetrahydroquinazolin-4-one (**8**) in moderate yield 62% [18]. This compound was then refluxed for three days with an excess of morpholine in dioxane. Evaporation of dioxane and addition of dichloromethane (DCM) resulted in precipitation of 8-bromo-2-morpholin-4-yl-3,4-dihydroquinazolin-4-one (**9**) which was collected by filtration in satisfactory yield (66%). Introduction of aryl/heteroaryl moieties (R^1) using aryl/heteroaryl boronic acids through Suzuki-Miyaura coupling with $Pd(OAc)_2$, 2-dicyclohexylphosphino-2′,4′,6′-triisopropylbiphenyl (XPhos), CsOH in combination with *n*-BuOH and H_2O as solvents afforded the first class of inhibitors (**10a-10 k**) in low to moderate yields, presumably ascribed to issues with purification (30–69%) [19]. Introduction of the polar heads started from 8-(4-nitrophenyl)-2-(morpholin-4-yl)-3,4-dihydroquinazolin-4-one (**11**). The nitro group of **11** was quantitatively reduced by zinc in acetic acid to the corresponding amino derivative (**12**), which was subsequently acylated with chloroacetyl chloride. The chloroamide product was collected by filtration and used without further purification in the next step, where the appropriate 6-membered saturated secondary amines or dialkylamines were attached enabling formation of the second family (**14a-e**) [13]. All of the 3,4-dihydroquinazolinones (**10a-k** and **14a-e**) displayed analytical and spectroscopic data in good agreement with their structures. Structural characterization involved melting points, NMR spectroscopy, and LC–mass spectrometry. Uncalibrated purity was ascertained by LC-UV using a reverse phase C18 chromatographic column. All the biologically tested compounds exhibited purity higher than 95% at different wavelengths. Detailed analyses for all the compounds are provided in the Supporting information.

2.2. Biological evaluation

Initially, the inhibitory effect of each compound alone was determined by WST-1 test after 48 h treatment. Single dose testing of growth inhibition on the screening panel of 16 human cancer and one healthy cell lines was performed with inhibitors at a concentration of 10 μmol/L. As a positive control, DOX was used at a concentration of 1 μmol/L. The results showed that derivative **10k** had the highest inhibitory effect, with proliferation in three out of the 17 cell lines reduced below 50%, and in 12 cell lines below 75%. **10e** and **10f** were other inhibitors with a significant antiproliferative effect (Table 1). In the light of these results, the colorectal adenocarcinoma cell line HT-29 was chosen as the most suitable model for further evaluation, as none of the tested inhibitors significantly affected proliferation of this cell line. Detailed results are provided in the Supporting information.

In the next step, the ability to potentiate the effects of the standard



Scheme 1. Synthesis of 3,4-dihydroquinazolinone derivatives **10a-k** and **14a-e**. Reagents and conditions: (a) $SOCl_2$, reflux, 2 h; (b) NH_4SCN, acetone, rt, 45 min, yield: 62%; (c) morpholine, dioxane, reflux, 72 h, yield: 66%; (d) Aryl boronic acids (R^1), $Pd(OAc)_2$, XPhos, CsOH, *n*-BuOH/H_2O 4:1, 50 °C, 2–24 h, yields: 30–69%; (e) Zinc, AcOH, rt, 12 h, yield: 95%; (f) $CH_2ClCOCl$, 0–25 °C, 12 h; (g) secondary amines (R^2), THF, 50 °C, 12 h, yields: 42–56%.

Table 1
Growth percentage in quinazolin-4-one series.

Code	R^1	R^2	GP[a] alone	GP[b] with DOX in HT-29 cells
10a	phenyl	–	85	59
10b	dibenzothien-4-yl	–	80	57
10c	thiophen-3-yl	–	86	61
10d	naphthalen-1-yl	–	86	48
10e	4-phenoxyphenyl	–	84	48
10f	dibenzofuran-4-yl	–	79	47
10g	4-methylphenyl	–	91	54
10h	(1,1′-biphenyl)-3-yl	–	88	33
10i	4-*tert*-butylphenyl	–	96	51
10j	7-chloroquinolin-4-yl	–	101	38
10k	5-methyl-1*H*-indol-3-yl	–	63	37
14a	–	4-methylpiperazin-1-yl	89	29
14b	–	morpholin-4-yl	85	24
14c	–	piperidin-1-yl	79	10
14d	–	diethylamino	77	8
14e	–	bis(2-hydroxyethyl) amino	71	36

[a] Growth percentage – the percentage of viable cells in all cell lines compared to untreated cells (100%). Data are the mean of three independent experiments.

[b] Growth percentage – the percentage of viable cells from HT-29 cell line compared to untreated cells (DOX alone GP = 58). Data are the mean of three independent experiments.

chemotherapeutic agent DOX was tested. Since DNA-PK is a key enzyme in DSBs repair mediated by NHEJ, DOX, a topoisomerase II-stabilizing drug that induces DNA DSBs was chosen as a model chemotherapeutic agent in this study. HT-29 cells were exposed to 10 μmol/L (standard concentration according to NCI-60 Screening Methodology) of the quinazoline derivatives in combination with 0.5 μmol/L DOX for 48 h (Fig. 2, Table 1). This concentration of DOX was determined by viability assessment: 0.5 μmol/L caused a decrease in cell viability to 58% relative to the proliferation of untreated control cells (100%). The standard DNA-PK inhibitor NU7441 at a concentration of 1 μmol/L was included in the screening as a positive control. In 2006, Zhao and colleagues studied chemosensitization by the specific DNA-PK inhibitor NU7441 (1 μmol/L) in human colon cancer cells LoVo and SW620. It was found that NU7441 markedly enhances the cytotoxicity of DOX and etoposide in both cell lines in a dose-dependent manner [20]. In 2014 Ciszewski et al. reported the synergic effect of NU7441 with DOX in breast cancer cell lines [21]. Within our work, a significant chemosensitizing effect in HT-29 cells by 13 out of 17 tested compounds (10 μmol/L) in combination with DOX (0.5 μmol/L) was also observed, highlighted by **14c** and **14d** which both led to rapid cellular inhibition. This finding was confirmed by the trypan blue exclusion technique.

The first subset, structurally mimicking NU7441, was expected to possess pronounced inhibition potency compared to others, especially those derivatives containing bulky moieties such as dibenzothiophene (**10b**) and dibenzofuran (**10f**) in the aromatic region. Instead, the most favorable substitutions improving chemosensitizing properties were noticed in biphen-3-yl-(**10h**), 7-chloroquinolin-4-yl-(**10j**) and 5-methyl-1*H*-indol-3-yl-containing (**10k**) analogues. Small aromatic substituents were not tolerated since the derivatives having phenyl (**10a**), thiophen-3-yl (**10c**), methylphenyl-4-yl (**10g**) or *tert*-butylphenyl-4-yl (**10i**) were almost ineffective. In contrast, the second family of derivatives exhibited significantly increased chemosensitizing properties on cells, which was not associated with stronger DNA-PK inhibition. Accordingly, the best secondary amines in terms of the highest antiproliferative action were piperidin-1-yl-(**14c**) and diethylamino-(**14d**) substituted derivatives. These two derivatives significantly surpassed other three derivatives in this series containing *N*-methylpiperidine-1-yl, morpholine-4-yl and (2-bishydroxyethyl)amine. From these results, we can suggest that only aliphatic or possibly other non-polar

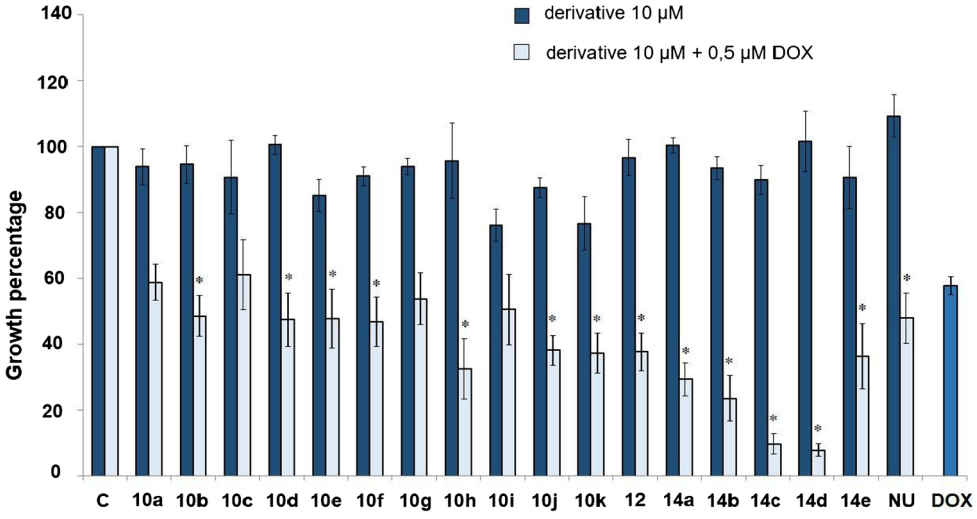


Fig. 2. Viability representation of HT-29 cell line after treatment of newly synthesized inhibitors (**10a-k** and **14a-e**; 10 μmol/L) or standard inhibitor NU7441 (NU; 1 μmol/L) with DOX (0.5 μmol/L) after 48 h of incubation. To calculate the cell viability, the value of the signal from the treated culture well was expressed as a percentage of that of the control well. Data are the mean of three independent experiments ± standard deviation. * P ≤ 0.05, versus DOX alone.

substitutions are well-tolerated on amine at position 2- of acetamide linker.

DOX is known to kill cancer cells through induction of apoptosis, or more rarely through autophagy or senescence [22]. To evaluate the mode of cell death induced by a combination of DOX with the most efficient sensitizer **14d**, we evaluated the activation of caspases, which are typical for apoptosis. Application of the inhibitor in combination with DOX caused induction of apoptosis that was revealed by Caspase-Glo Assay 48 h after the treatment. A significant increase was found (P ≤ 0.05) in the activities of caspase 3/7 and caspase 9 when **14d** or the standard DNA-PK inhibitor NU7441 was combined with DOX (in comparison with the effect of DOX alone). The activity of caspase 8 was significantly increased only in the combination of inhibitor **14d** with DOX (Fig. 3). We may conclude that initiator caspases-8 and -9 were activated by dimerization from inactive procaspase monomers after combination therapy. Activated initiator caspases then cleaved inactive procaspase dimers of executioner caspases-3/7. Once activated, a single executioner caspase can cleave and activate other executioner caspases. This process lead to an accelerated feedback loop of caspase activation and apoptotic cell death [23].

In addition, we used flow cytometry analysis to inspect whether inhibition of DNA-PK activity affects the cell cycle phases in HT-29 cells. We found that the effect of the cytotoxic agents alone as well as in combination with **14d** caused an accumulation in G2 phase (Fig. 4), similarly as in combination with the standard DNA-PK inhibitor NU7026 at the same concentration. The cell cycle is arrested due to activation of the G2/M checkpoint which prevents or delays mitosis in the presence of DNA lesions [24]. This is consistent with the

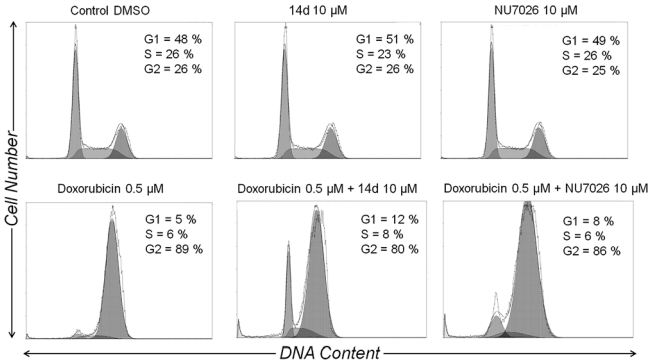


Fig. 4. Effect of chemical agents on the cell cycle after 24 h. The numbers in the histograms represent the percentage of cell cycling through phases G1, S, and G2 of the cycle. The cell cycle profiles are from a single representative experiment.

observation made by Zhao et al. who tested DNA-PK inhibitor NU7441 combined with DOX and etoposide in SW620 cells [20]. In 2014, Pastwa et al. also noticed G2/M arrest after application of wortmannin and cisplatin/etoposide in human glioblastoma cell line M059 [25]. On the other hand, there were no statistically significant differences in the cells affected by combination therapy in comparison with DOX administered alone.

Protein p53 has an essential function in the cellular response to DNA damage, serving as ATM substrate. The function of protein p53 is regulated by a number of mechanisms. One such is the important role of

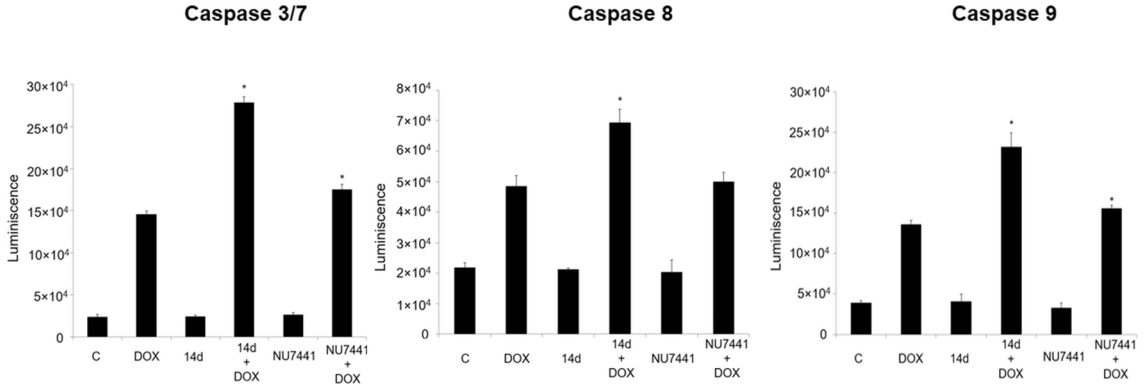


Fig. 3. Activity of caspases 3/7, 8 and 9 was determined 48 h after treatment (DOX 0.5 μmol/L, **14d** 10 μmol/L, NU7441 1 μmol/L) in human colorectal adenocarcinoma cells HT-29. Results are shown as mean ± standard deviation from three measurements. * P ≤ 0.05, versus DOX alone.

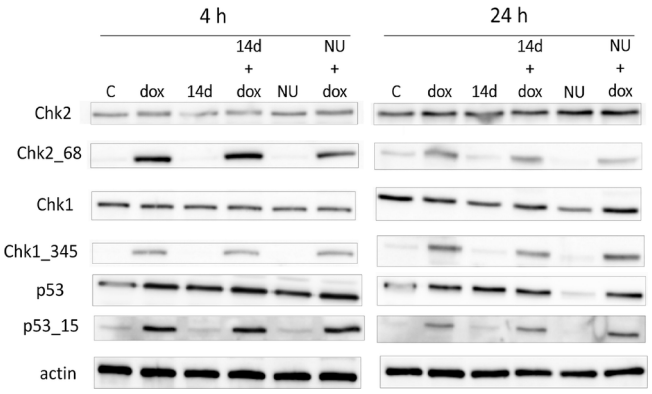


Fig. 5. Induction and activation of proteins in cells treated with DOX (0.5 μmol/L), inhibitors **14d** and NU7026 (10 μmol/L), and combinations in human colorectal adenocarcinoma cell line HT-29 after 4 and 24 h. To confirm equal protein loading, membranes were incubated with *β*-actin. Data are representative for one of three independent experiments.

Mdm2 protein for effective p53 degradation. When cells are exposed to DSB-inducing stress that cannot be repaired, ATM causes phosphorylation of Mdm2 and p53 on Ser15. Increasing the phosphorylation on Ser15 means that ATM kinase is not inhibited [26,27]. In our experimental procedure, DOX alone, DOX in combination with **14d**, and DOX with NU7026 all caused a significant up-regulation in p53 (Fig. 5). Activation and stabilization of p53 is connected with a series of post-translational modifications. Here we observed phosphorylation of p53 at Ser15 after the treatment with DOX alone, as well as in combination with inhibitors **14d** and NU7026. Interestingly, while NU7026 alone caused only transient increase in p53 amount, application of inhibitor **14d** caused an increase in p53 amount lasting for the whole duration of the experiment. This induction was not accompanied by phosphorylation on serine 15.

DOX alone and in combination with both inhibitors (**14d** or NU7026) provoked phosphorylation of Chk2 on Thr68 and Chk1 on Ser345, and therefore ATM and ATR pathways were not affected. These phosphorylated checkpoint kinases are elevated in response to DNA damage, where ATR and ATM kinase substrates Chk1 and Chk2 respectively allow cells to delay progression through the cell cycle, repair the DNA, and possibly induce cell death [28]. Chk1 is known to be activated by ATM/ATR kinase-mediated phosphorylation at Ser345, which in turn phosphorylates Cdc25A/C and finally causes cell arrest in the late S or G2 phase. The activation of Chk2 in checkpoint signaling is initiated by phosphorylation at Thr68 in ATM/ATR-dependent manner. In addition to the canonical role of Chk1 and Chk2 in cell cycle checkpoint regulation, recent discoveries have underlined the roles of both kinases in controlling DNA repair and apoptosis [29]. Inhibition of checkpoint kinase activation abrogates cell cycle arrest, forcing cells to enter mitosis prior to completion of DNA damage repair [30,31].

The most promising compounds **14c**, **14d** and NU7441 analog **10b** were chosen for advanced biological assays to screen inhibitory potency against DNA-PK, ATM, ATR and various isoforms of PI3K by Eurofins Pharma Discovery Services UK Limited. The derivative **10b** is not very active as a chemosensitizer to DOX; however, it possesses structural features resembling DNA-PK inhibitor NU7441. It was therefore chosen for the determination of its inhibition potency to confirm whether its low activity is a consequence of weak DNA-PK inhibition or is caused by cell-associated issues (permeability, efflux from cells). The results confirmed that **10b** does not affect ATM or ATR, but does however possess DNA-PK affinity in the micromolar range. Indeed, **10b** had IC_{50} value of 6.0 μmol/L and **14d** 12.4 μmol/L, while the activity of **14c** was not even determined at concentration of 30 μmol/L. PI3K isoforms were also not affected by these selected compounds (Table 2, Supplementary Information, Table S3).

Table 2
Results of Eurofins IC_{50} Profiler™ study of derivatives **10b** and **14d**.

Compound	Target kinase	IC_{50} (μmol/L)
10b	ATM	> 30
	ATR	29.6
	DNA-PK	6.0
	PI3K*α*	> 30
	PI3K*β*	> 30
14d	ATM	> 30
	ATR	> 30
	DNA-PK	12.4
	PI3K*α*	> 30
	PI3K*β*	> 30

These results were unexpected. Positive potentiation of DOX toxicity was assumed to be ascribed to inhibition of DNA-PK, but instead it seems that several off-targets are responsible for their high DOX potentiation. With respect to these results, we submitted compound **14d** for evaluation on a broad oncology panel containing 104 different protein kinases in order to reveal its novel targets. However, only DNA-PK, Aurora-A, Pim-1, Flt4, and cKit (V560G) proteins were inhibited below 50% at 10 μmol/L of **14d**. Under these conditions, DNA-PK activity was suppressed to 36%, suggesting that the IC_{50} value could be even lower than the initially established value of 12.4 μmol/L. Nevertheless, the inhibition activity towards DNA-PK is still considered as relatively weak. In order to elucidate the specific target for **14d**, we have applied an *in silico* off-target screening approach (see Supplementary Information, Table S4). This technique can help to identify a completely diverse group of target proteins responsible for the effect observed *in vitro* [32]. Briefly, Protein Data Bank (PDB) receptors from 2013 were utilized to uncover plausible off-targets of **14d**. Initially, ligand was docked to all ~9000 proteins. After that, the 40 top-scored hits were selected for detailed calculations [33]. From the molecular modeling study, poly(ADP-ribose) polymerase-1 (PARP-1) has emerged as the potential target of **14d**. PARP-1 is one of the proteins from an 18–membered superfamily implicated primarily in the DNA repair mechanism, specifically in base excision repair and repair of DNA single-strand breaks (SSBs) [34,35]. In the recent study by Park and co-workers, combined use of the PARP-1 inhibitor olaparib (an FDA-approved anti-cancer agent for the treatment of BRCA1/BRCA2-deficient carcinomas) and DOX synergistically inhibited proliferation of various types of osteosarcoma cells [36,37]. Similarly, another PARP inhibitor, namely NU1025, enhanced the cytotoxicity of bleomycin or DNA-methylating agent MTIC [38]. The structurally similarity of NU1025 to novel quinazolin-4-one derivatives goes hand in hand with the findings from *in silico*. Accordingly, the high *in vitro* efficacy of **14d** with DOX allowed us to hypothesized that its effect is mediated via DNA-PK and PARP–1 inhibition. PARP-1 is also known to target p53. PARylation of p53 inhibits export of p53 from the nucleus [39], contributing to its accumulation and also supporting the transactivation role of p53 [40]. This could explain prolonged accumulation of p53 without phosphorylation of serine 15, which was induced by **14d**.

From the results of the chemosensitization study, it is evident that the mode of action of the compounds is not exclusively attributed to blockade of DNA-PK. Thus, the action of 3,4-dihydroquinazolin-4-one derivatives as chemosensitizing agents is corroborated via multiple, not disclosed targeting. Surprisingly, the intended solubility improvement by polarizing the aromatic end of the molecule led to highly potent agent. With this respect, the activity of **14d** in combination with DOX is remarkable. The crucial part of our work in the future will be devoted to evaluating the molecular targets of **14d**.

The safety and tolerability of **14d** were established in female mice. The animals were observed for clinical score and weight loss (the limits were 10% or 15%, respectively) over 5 days after *i.p.* injection of a solution of the compound. Some authors such as Aston and colleagues

use a level of 15%, but 10% can be seen as a step towards refinement of the MTD [41].

In our experimental protocol, the doses were 5, 10, 15, 20, 25, and 30 mg/kg (the highest dose available due to the limited solubility of the compound). None of the doses of **14d** gave rise to any apparent clinical symptoms nor to body loss of more than 10%, except in the case of one mouse given 30 mg/kg. The said mouse lost 15% of its weight by day 3, but recovered until the end of the experiment. It can be concluded that 30 mg/kg corresponds to MTD (Table S2). Before this dose can be considered as the highest tolerated dose in a future preclinical study, potential differences between mouse strains have to be taken into account [42].

3. Conclusion

In this paper, we described the design and synthesis of novel quinazolin-4-one derivatives with potential chemosensitizing activity to DOX cytotoxicity on different cancer cell lines. Biological assessment showed a chemosensitizing effect of compound **14d** with the standard anticancer agent DOX, with a strong effect on the proliferation of human colorectal carcinoma HT-29 cells. This effect was caused by caspase-dependent apoptosis. We confirmed that this process was not initiated by the ATM/ATR pathway. The chemosensitizing effect of **14d** seems to be the synergistic action of DNA-PK inhibition, proved *in vitro*, with plausible PARP-1 inhibition, indicated *in silico*. In monotherapy, most of the compounds did not significantly affect the cell growth of 16 human cancer cell lines, including non-carcinoma human dermal fibroblasts. The lead compound **14d** exerted a relatively low toxicity *in vivo* (MTD = 30 mg/kg) when administered to female mice. Taken together, this work will also aid the design of further chemosensitizing ligands with increased kinase specificity and selectivity, and help to determine their true therapeutic utility.

Conflict of interest

The authors declare no competing interests.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2018.10.001.

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