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

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ARTICLE INFO

Keywords:
DNA-dependent protein kinase
Poly(ADP-ribose) polymerase-1
Phosphatidylinositol 3-kinase
NU7441
Cancer
Chemosensitization

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 chemosensitizing 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) [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′-trisopropylbiphenyl

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https://doi.org/10.1016/j.bioorg.2018.10.001
Received 1 August 2018; Received in revised form 1 October 2018; Accepted 2 October 2018
Available online 06 October 2018
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Core scaffold modification was another extensively pursued possibility to obtain novel lead compounds. Accordingly, quinolinones (4), pyrido[1,2-α]pyrimidinones (5) and 1,3-benzoazinones (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].

### 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 (R1) using aryl/heteroaryl boronic acids through Suzuki-Miyaura coupling with Pd(OAc)$_2$, 2-dicyclohexylphosphino-2',4',6'-trisopropylbiphenyl (XPhos), CsOH in combination with n-BuOH and H$_2$O as solvents afforded the first class of inhibitors (10a-10k) 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 48h 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
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-1H-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 anti-proliferative 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

**Table 1**

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

$^a$ 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.
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 48h 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 caspsases-8 and -9 were activated through caspase-8 activation after the initiator caspase is 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 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...
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 IC50 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

<table>
<thead>
<tr>
<th>Compound</th>
<th>Target kinase</th>
<th>IC50 (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10b</td>
<td>ATM</td>
<td>&gt;30</td>
</tr>
<tr>
<td></td>
<td>ATR</td>
<td>29.6</td>
</tr>
<tr>
<td></td>
<td>DNA-PK</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>PI3Ka</td>
<td>&gt;30</td>
</tr>
<tr>
<td></td>
<td>PI3Kβ</td>
<td>&gt;30</td>
</tr>
<tr>
<td>14d</td>
<td>ATM</td>
<td>&gt;30</td>
</tr>
<tr>
<td></td>
<td>ATR</td>
<td>&gt;30</td>
</tr>
<tr>
<td></td>
<td>DNA-PK</td>
<td>12.4</td>
</tr>
<tr>
<td></td>
<td>PI3Ka</td>
<td>&gt;30</td>
</tr>
<tr>
<td></td>
<td>PI3Kβ</td>
<td>&gt;30</td>
</tr>
</tbody>
</table>

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 IC50 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-dihydroxquinazolin-4-one derivatives as chemosensitizing agents is corroborated via multiple, not disclosed targeting. Surprisingly, the intended solubility improvement 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 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 with plausible PARP-1 inhibition, indicated

Conflict of interest

The authors declare no competing interests.

Funding

This work was supported by Charles University project GAUK 932516 and programs Progr. Q40/01 and SVV-260397/2017; by University of Defence specific research project SV/FV2021402 and Long Term Development plan of Faculty of Military Health Sciences; by MH CZ – DRO (University Hospital Hradec Kralove, No. 00179906); by Technology Agency of the Czech Republic project no. TG02010020. MH CZ – DRO (University Hospital Hradec Kralove, No. 00179906); by Long Term Development plan of Faculty of Military Health Sciences; by 932516 and programs Progres Q40/01 and SVV-260397/2017; by

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