CHEMISTRY & BIODIVERSITY

Accepted Article

Title: Nucleolipids of the Nucleoside Antibiotics Formycin A and B: Synthesis and Biomedical Characterization particularly using Glioblastoma Cells

Authors: Helmut Rosemeyer, Christine Knies, Katharina Hammerbacher, Eugenia Bender, Gabriel A. Bonaterra, Ricarda Hannen, Jörg W. Bartsch, Christopher Nimsky, and Ralf Kinscherf

To be cited as: *Chem. Biodiversity* 10.1002/cbdv.201900012

Link to VoR: http://dx.doi.org/10.1002/cbdv.201900012

Nucleolipids of the Nucleoside Antibiotics Formycin A and B: Synthesis and Biomedical Characterization particularly using Glioblastoma Cells[#])

Helmut Rosemeyer [1]), Christine Knies [1]), Katharina Hammerbacher [2]), Eugenia Bender [1]), Gabriel A. Bonaterra[2]), Ricarda Hannen [3]), Jörg W. Bartsch [3]), Christopher Nimsky [3]), and Ralf Kinscherf [2])

[1]) Organic Chemistry I - Bioorganic Chemistry, Institute of Chemistry of New Materials, University of Osnabrück, Barbarastr. 7, D-49069 Osnabrück

[2]) Institute for Anatomy and Cell Biology, Department of Medical Cell Biology, Philipps-University Marburg, Robert-Koch-Str. 8, D-35032 Marburg

[3]) Department of Neurosurgery, Philipps-University Marburg, Baldingerstr. D-35032 Marburg, Germany

E-Mail: Helmut.Rosemeyer@uos.de

[#])Dedicated to the memory of the late*Klaus Mühlegger*, Penzberg and Polling, Bavaria, Germany

Abstract. Two lipophilic derivatives of formycin A (**1**) and formycin B (**5**) carrying an O-2',3'-ethyllevulinate ketal group have been prepared. These were base-alkylated at N(1) (for **1**) and N(1) and N(6) (for **5**) with both, isopentenyl or all-*trans* farnesyl residues. Upon the prenylation side reactions were observed, resulting in the formation of nucleolipids with a novel tricyclic nucleobase (→ **4a**,**b**). In case of formycin B O-2',3'-ethyllevulinate (**6**) farnesylation gave the double prenylated nucleolipid **7**. All new compounds were characterized by ^{1}H-, ^{13}C-, UV-Vis, and fluorescence spectroscopy, by ESI mass spectrometry and/or by elemental analysis.Log*P* determinations between water and n-octanol as well as water and cyclohexane of a selection of compounds allowed qualitative conclusions concerning their potential blood-brain barrier passage efficiency. All compounds were investigated *in vitro*with respect to their cytotoxic activity toward rat malignant neuroectodermal BT4Ca as well as against a series of human glioblastoma cell lines (GOS 3, U-87 MG and GBM 2014/42). In order to differentiate between anticancer and side effects of the novel nucleolipids, we also studied their activity on PMA-differentiated human THP-1 macrophages. Here we show, that particularly the formycin A derivative **3b** possesses promising antitumor propertiesin several cancer cell lines with profound cytotoxic effects partly on human glioblastoma cells, with a higher efficacy than the chemotherapeutic drug 5-fluorouridine.

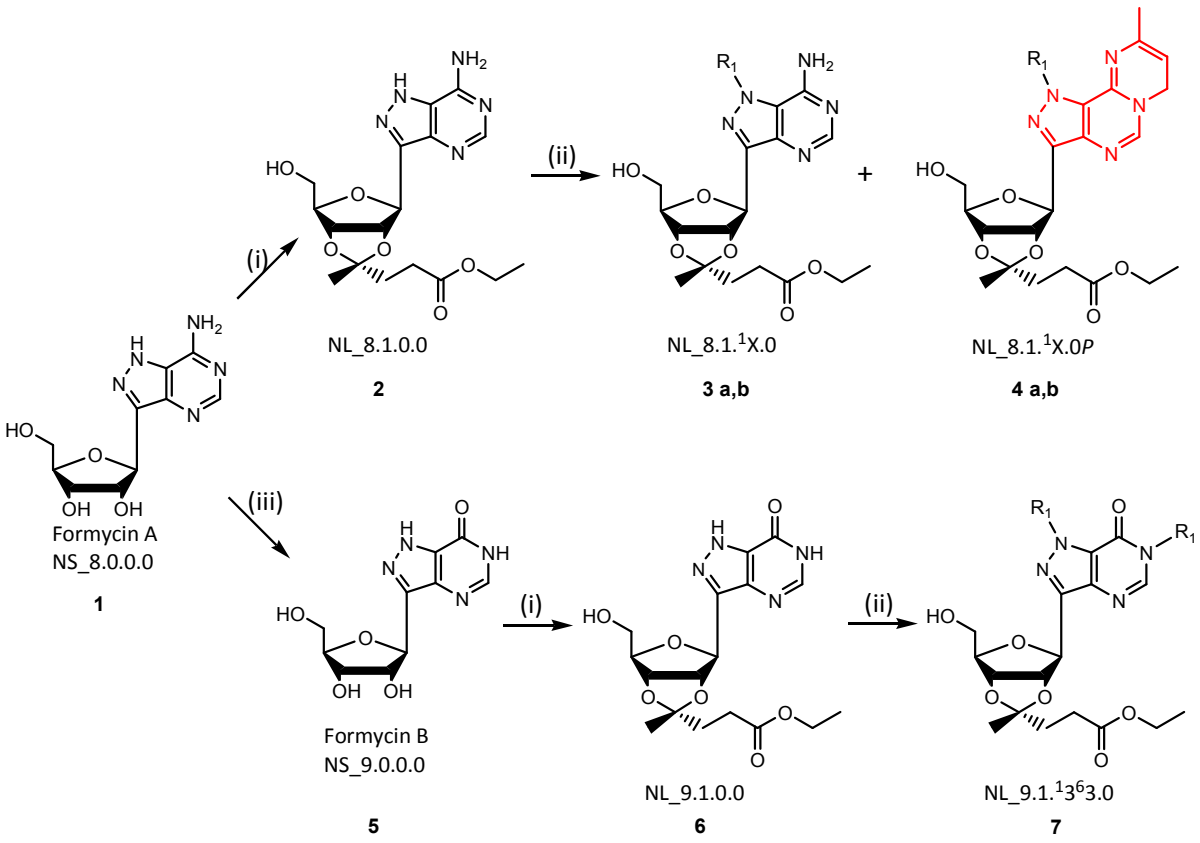
Key words: Formycin A and B, Nucleolipids, Drug Profiling, Glioblastoma

1. Introduction. The C-nucleoside antibiotic formycin A (from *Nocardia interforma*), an adenosine analogue, is a fluorescent nucleoside with an extremely stable glycosylic bond.For a comprehensive overview about formycin A and B, its chemistry, biochemistry and fluorescence spectroscopy, see [1]. Formycin A exhibits activity against various tumor cell lines such as Ehrlich carcinoma, HeLa cells,and Yoshida rat sarcoma [2].Besides the anti-tumoral impact of formycin A,an insulinotropic action was reported [3]. It is rapidly deaminated to its inosine analogue, formycin B, by the action of adenosine deaminase [1, 2].*J. Gizie*wicz et al.[4] synthesized a series of base- and ribose-methylated formycin A derivatives and tested their antiviral and antitumor activity. In the course of this work it turned out that N(1)-methylformycin A proved to be extraordinarily resistant towards the enzymatic deamination by adenosine deaminase. *F. Seela* et al. [5] prepared the N(1)-methylated 2'-deoxy-ß-D-ribonucleosides of formycin A and B and their 2'-cyanoethylphosphoramidites as well as an N(1)-carboxymethyl-functionalized formycin A compound and digoxigenin derivatives thereof for the detection of nucleic acids [6].

In this paper we report on the preparation of nucleolipids of formycin A(**1**, NS_8.0.0.0) and B (**5**, NS_9.0.0.0)and similar tricyclic formycin A derivatives carrying additional lipophilic moieties at N(1) and exhibiting strong fluorescence.Formycin B (**5**, NS_9.0.0.0) was prepared from formycin A by deamination with adenosine deaminase.

2. Syntheses of Formycin A and B Compounds. Reaction of formycin A (**1**) with ethyllevulinate in the presence of triethylorthoformate and 4M HCl in 1,4-dioxane in dry and amine-free DMF gave – after 24 h – the O-2',3'-ketal derivative **2** as main product which was purified by column chromatography. Subsequent reaction of **2** with *trans*,*trans*-farnesyl bromide in the presence of K_2CO_3 in DMF gave compound **3b** as main product besides a small amount of a highly blue-fluorescent, faster migrating by-product, in the following identified as compound **4b**(Formula Scheme 1) - formed upon prenylation- carrying a novel

heterocyclic system. Both compounds were analyzed by ^{1}H-(see *Supplementary Material*) and ^{13}C-NMR spectroscopy; assignment of the resonances was made with the help of DEPT-135, [^{1}H,^{1}H]- and [^{1}H,^{13}C]-correlation spectroscopy. Moreover, ESI mass spectrometry as well as UV- and fluorescence spectroscopy (see *Supplementary Material*) proved the structure and integrity of the compounds.



Formula scheme 1. Syntheses of Formycin A- and Formycin B-Nucleolipids. (i) DMF, Ethyllevulinate, $HC(OEt)_3$ H^+/1.4-Dioxane; (ii) at FA: DMF, K_2CO_3, X = 1 = R^1 = Isopentenylbromide, X = 3 = R_1 = Farnesylbromide; (ii) at FB: DMF, K_2CO_3, R_1 = Farnesyl-; (iii) H_2O, ADA. Red colored new heterocyclic system: 1,7-dihydropyrazolo[3,4-e]pyrimido[1,2-c]pyrimidine (red highlighted).

Formycin B (**5**) was prepared from formycin A (**1**) by deamination with adenosine deaminase. Of particular interest was the detection of compound **4b**, the formation of which is conceivable by reaction of compound **3b**with an excess of farnesyl bromide by an unknown mechanism. In Formula Scheme S2 (*Supplementary Material*) two variants of

thisside reaction are tentatively postulated.Compound **4b** contains a novel N-heterocyclic ring system, namely a 9-methylated 1,7-dihydropyrazolo[3,4-*e*]pyrimido[1,2-*c*]pyrimidine carrying a glyconic residue with an O-2',3'-ethyllevulinate moiety at O-2',3' and a *trans,trans*-farnesyl residue at N(1). A reaction of compound **2** with isopentenyl bromide gave two analogous reaction products (compounds **3a** and **4a**).

Both farnesylated compounds (**3b**, **4b**) exhibit similar R_f values; the slightly faster migrating spot, however, shows a blue fluorescence upon irradiation at 366 nm, while the slower migrating nucleolipid does not. In both cases (isopentenylation, **3a**, **4a**; farnesylation, **3b**, **4b**) the products were separated chromatographically and characterized (see experimental part). A presentation of the corresponding TLC plates, of the UV/Vis spectra, the ESI mass spectra as well as of the ^{1}H-NMR spectra can be found in the *Supplementary Material* Section (S1-S6). Figure 1 shows an energy-minimized computer model of compound **4b**formed upon prenylation; Figure 2presents an optical comparison of the fluorescence of compounds **1**, **2**, **3b**, **4b**and **4a** (0.1 mM in methanol) upon irradiation at 254 nm.

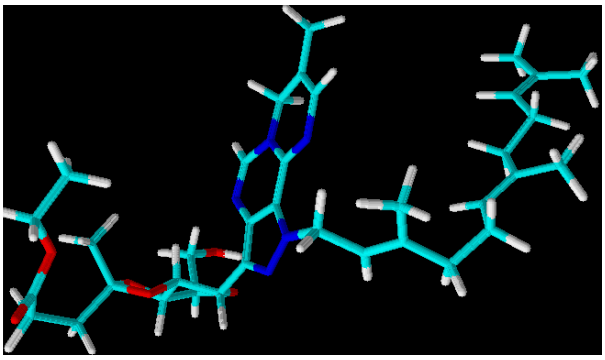


Figure 1. Computer Modelling of Compound NL_8.1.^{1}3.0*P* (**4b**, Formula Scheme 1).

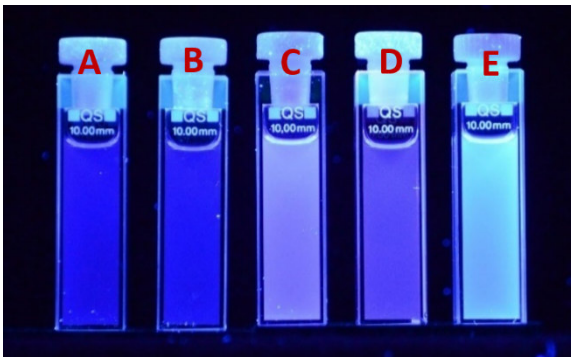


Figure 2. Comparison of fluorescence at a concentration of 0.1 mM in MeOH. Irradiation 254 nm, cut-off filter: 280 nm. A: formycin A (**1**), B: NL_8.1.0.0 (**2**), C: NL_8.1.^{1}3.0 (**3b**), D: NL_8.1.^{1}3.0*P*(**4b**), E: NL_8.1.^{1}1.0*P*(**4a**).

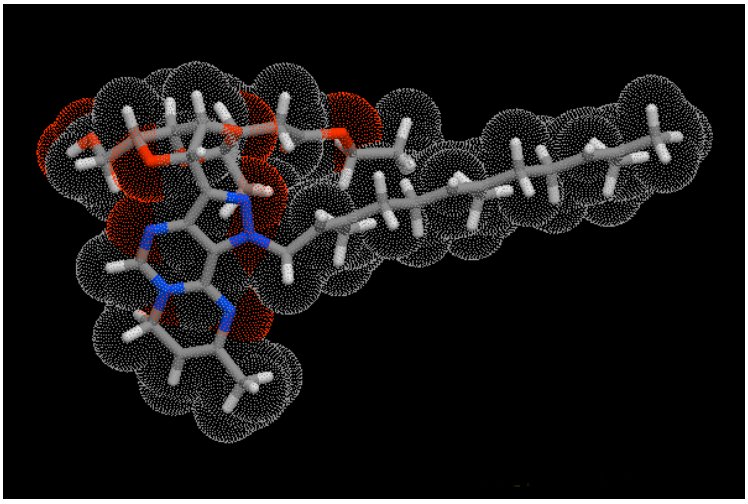


Figure 3. Presentation of the topological polar surface area of compound **4b**.

Figure 3 displays the topological surface area of compound **4b**which amounts to 126.96 Å^2. According to *D. E. Clark* [7] and *M. D. Wessel* [8] this value lies in a range which is acceptable for an intestinal drug absorption.

3. Biophysical Studies.

In order to evaluate how much lipophilization of formycin A (**1**) changes its lipophilicity, the $^{10}\log P_{OW}$ values of all nucleolipids were determined *in silico* using the http://eadmet.com/de/physprop.php website with *ePhysChem* that contains ALOGPS v.3.0. [9,10]. In addition, the values of NS_8.0.0.0 (**1**) and NL_8.1.^{1}3.0P (**4a**)were determined experimentally (see experimental. part). Moreover, the corresponding $^{10}\log P_{Ch}$ data of the latter nucleolipids were measured in water-cyclohexane (Table 1). According to the work of *P. Seiler* [11] and later of *R. C. Young* [12], the difference of the two $^{10}\log P$valuesallows a rough assessment of the ability of various low-molecular-weight drugs to cross the blood-brain barrier, applying eq. 1:

$$^{10}\log(C_{Brain}/C_{Blood}) = -0.604\ (\pm\ 0.169)\ \Delta^{10}\log P + 1.23\ (\pm\ 0.56) \quad \text{(eq. 1)}$$

Table 1. Biophysical data of NS_8.0.0.0(**1**) and its nucleolipids NL_8.1.^{1}3.0 (**3b**) andNL_8.1.^{1}3.0*P*(**4b**).

Compound	log*S*	logP_{OW} (calc.)	logP_{OW} (measured)	logP_{ChW} (measured)	logP_{OW}-logP_{ChW}	C_{Brain}/C_{Blood}
NS_8.0.0.0, **1**	-1.28	-1.25 ± 0.74	-2.11	-2.29	0.185	13.13
NL_8.1.^{1}3.0, **3b**	-4.93	2.78 ± 0.74	2.20	2.06	0.140	14.00
NL_8.1.^{1}3.0*P*, **4b**	-4.34	3.05 ± 0.74	2.83	2.69	0.140	14.00

S: water solubility

Inspection of the data shown in Table 1 exhibits a slighttheoretical improvement of the blood-brain passage of Formycin A upon O-2',3'-ketalization with an ethyllevulinate residue and a further hydrophobization at the heterocyclic base. However, from Table 1 it can be seen that there is no difference in the C_{Brain}/C_{Blood} values between compounds **3b** and **4b**.

4. Biological Studies.

To differentiate anticancer-fromsideeffects [20], we treated either brain tumor cell lines or human macrophages as non-tumor cell line with compounds as indicated*in vitro.*As brain tumor cells, rat malignant neuroectodermal BT4Ca[13], human astrocytoma/oligodendroglioma GOS-3, human glioblastoma U-87MG (ATCC HTB-14) and patient-derived human primary glioblastoma cells GBM 2014/42 were studied. As non-tumor cell line, human THP-1 monocytic cells were used that had been differentiated with phorbol 12-myristate 13-acetate (PMA) to represent a macrophage phenotype. The effects of 5-fluorouridine (5-FUrd; NS_4.0.0.0 [14]; ~positive control), formycin A (NS_8.0.0.0, **1**) and its previously described derivativesNL_8.1.0.0 (**2**), NL_8.1.^{1}3.0 (**3b**), NL_8.1.^{1}3.0*P*(**4b**), NL_8.1.^{1}1.0*P*(**4a**)plus formycin B (NS_9.0.0.0, **5**), NL_9.1.0.0**6**), and NL_9.1.^{1}3^{6}3.0 (**7**)on cell viability were determined after a 48h incubation with thesenovel

compounds(Figures. 4-13).The experimental setting of our viability/cytotoxicity assays was chosen according to previous results using other novel anti-tumor derivatives, where we found 100 % cytotoxicity already at 50 µM after 48 h treatment (e. g. Farhat et al. 2015; Knies et al., 2015a, 2015b, 2016). Additionally, under these experimental conditions, active derivatives revealed an IC50 between 1.56 µM and 50µM in different tumor cell lines (e. g. Farhat et al. 2015; Knies et al., 2015a, 2015b, 2016).

After treatment (48h) of rat malignant neuroectodermal BT4Ca cells with NS_4.0.0.0 (5-FUrd), we observed a significant($p<0.001$) inhibition of cell viability at all concentrations testedby -66.3 % (1.56 µM), -69.3 % (3.12 µM), -75.4 % (6.25µM), -77.9 % (12.5µM), -81.9 % (25 µM) and -83.3 %(50 µM) compared to DMSO that was always used as control (~100% viability, Figures. 5& 6). Formycin A (**1**, NS_8.0.0.0) itself showed an almost concentration-dependent effect and diminished cell viability significantly by -23.9 % (12.5 µM, $p<0.05$), -46.7 % (25 µM, $p<0.01$) and -63.1 % (50 µM, $p<0.001$)compared to DMSO (Figure. 4).

Treatment of BT4Ca cells with NL_8.1.0.0 (**2**) at 25 µM resulted in a significant increase of cellviability by +16.1 % ($p<0.05$)compared to DMSO (Fig. 5).In contrast, at all concentrations tested,NL_8.1.^{1}3.0 (**3b**) significantly inhibited cell viability by -37.9 % (1.56 µM; $p<0.01$), -65.1 % (3.12 µM; $p<0.01$), -80.3 % (6.25 µM; $p<0.01$), -87.5 %(12.5 µM; $p<0.001$), - 92.4 % (25 µM; $p<0.001$) and -95.1 % (50 µM; $p<0.01$) compared to DMSO (Fig. 4).

In contrast, the derivative NL_8.1.^{1}3.0 *P*(**4b**) significantly enhanced cell viability by +18.2 % (1.56 µM, $p<0.001$), +20.0 % (3.12 µM, $p<0.01$), +32.9 % (6.25 µM, $p<0.001$) and + 13.7 % (12.5 µM, $p<0.001$) compared to DMSO. Only at a concentration of 50 µM,NL_8.1.^{1}3.0*P*(**4b**) revealed a significant ($p<0.001$) inhibition of cell viability by -75.8 %

compared to DMSO (Fig. 5). In addition, derivative NL_8.1.^{1}1.0 *P*(**4a**) showed at 50 µM a significant ($p<0.05$) reduction of the viability by -10.6 % compared to DMSO (Fig. 4).

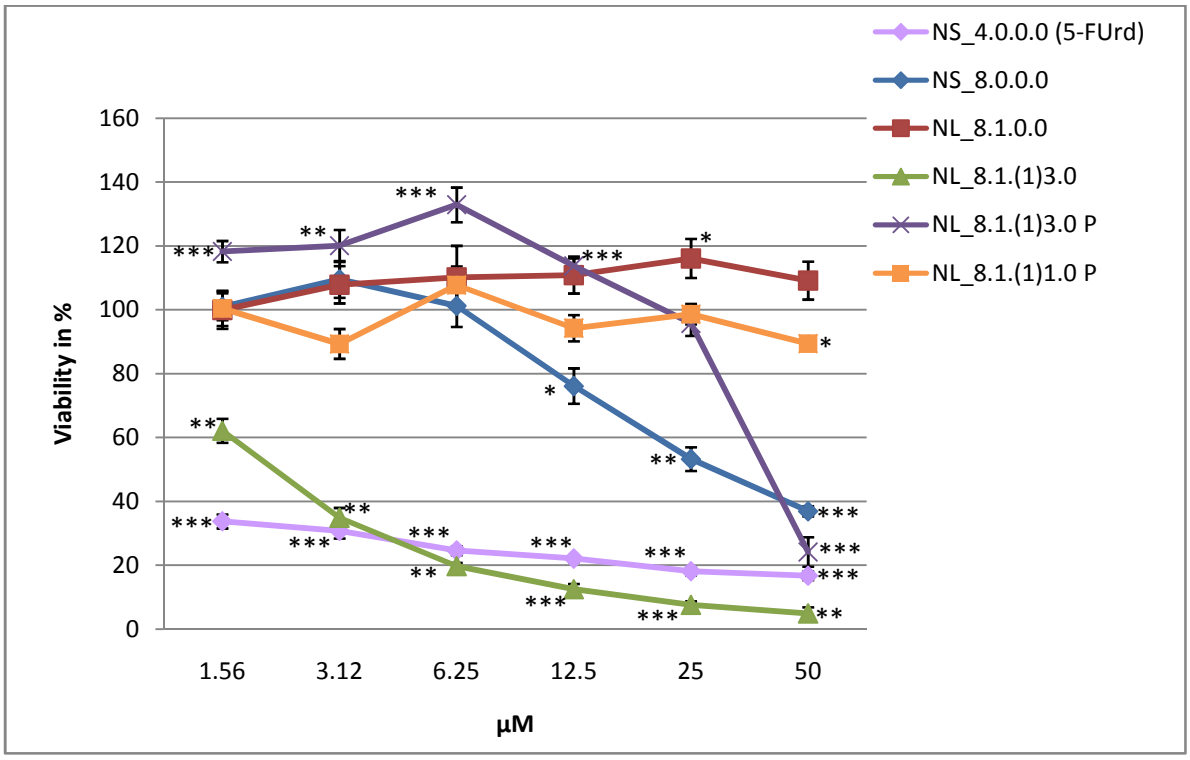


Figure 4.Viability (in %) of rat BT4Ca astrocytoma/oligodendroglioma cells after incubation (48h) with NS_4.0.0.0 (5-FUrd), Formycin A (NS_8.0.0.0**1**) or its derivatives (NL_8.1.0.0, **2**, NL_8.1.^{1}3.0, **3a**, NL_8.1.^{1}3.0*P*, **4a**, and NL_8.1.^{1}1.0*P,* **4b**) is shown. Values are given (in % viability of control [incubation with medium alone = 100 % viability]) as mean ± SEM; *p<0.05, **p<0.01, ***p<0.001, significance vs. DMSO control (equal concentration of DMSO to 50 µM substance solution), n=5 independent experiments assayed in quadruplicates.

Formycin B (NS_9.0.0.0, **5**) at a concentration of 12.5 µMsignificantly increased viability of BT4Ca cells ($p<0.05$) by +10.4 %compared to DMSO (Fig. 5).At the same concentration (12.5 µM), its derivative NL_9.1.^{1}3^{6}3.0 (**7**) also led to a significant ($p<0.05$) increase of cell viability by +17.9 %compared to the DMSO control (Fig.5), while derivative NL_9.1.0.0 (**6**) insignificantly diminished viability by -32.4 % (50 µM) (Fig.5).

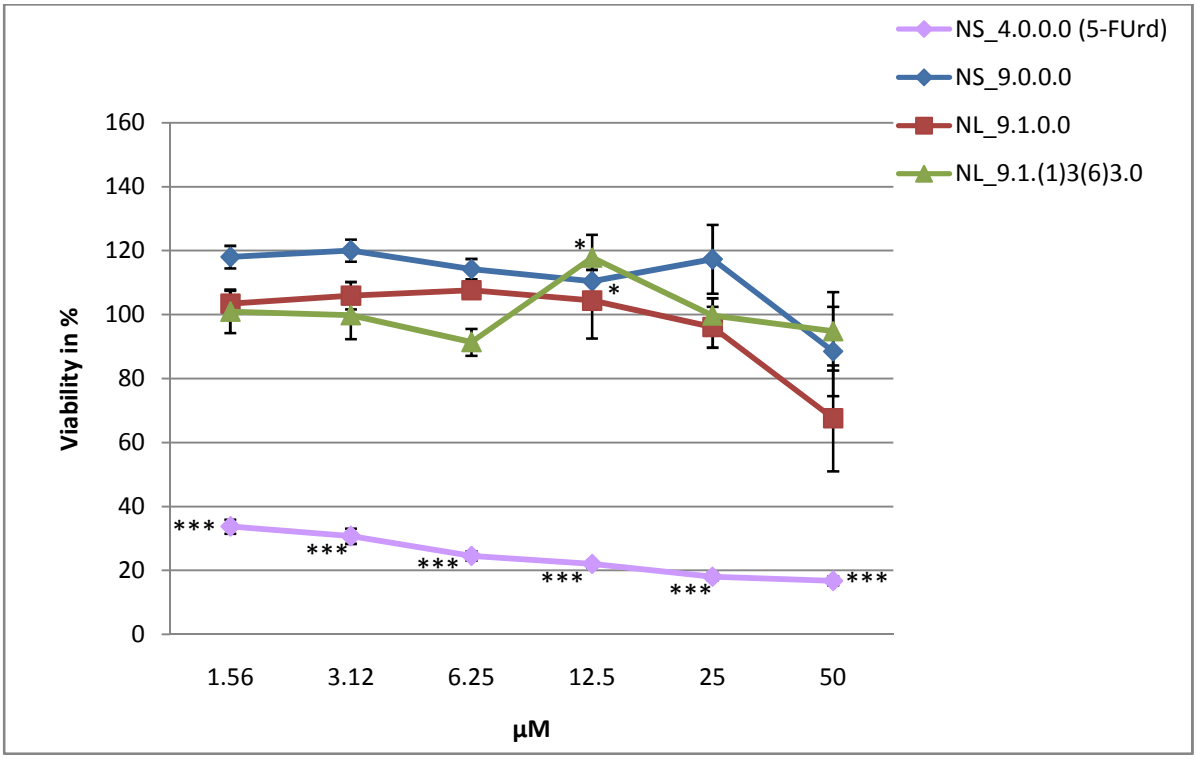


Figure 5.Viability (in %) of rat BT4Caastrocytoma/oligodendroglioma cells after incubation (48h) with NS_4.0.0.0 (5-FUrd), Formycin B (NS_9.0.0.0, **7**) or its derivatives (NL_9.1.0.0, **6,** and NL_9.1.^{1}3^{6}3.0, **7**) is shown. Values are given (in % viability of control [incubation with medium alone = 100 % viability]) as mean ± SEM; *p<0.05, **p<0.01, ***p<0.001, significance vs. DMSO control (equal concentration of DMSO to 50 µM substance solution), n=5 independent experiments assayed in quadruplicates.

Next, we tested the human GOS-3 glioblastoma cells in response to the compounds. Treatment (48h) of GOS-3 cellswith NS_4.0.0.0 (5-FUrd)resulted in a significant reductionof cell viability (Figs.6, 7). 5-FUrd significantly decreased the viabilityat all concentrations testedby -16.7 % (1.56 µM; p<0.01), -15.9 % (3.12 µM; p<0.05), -20.0 % (6.25 µM; p<0.001), -20.1 % (12.5 µM; p<0.001), -26.4 % (25 µM; p<0.001) and -29.7 %(50 µM; p<0.001) compared to DMSO (~100% viability) (Figs. 6, 7).

Formycin A (NS_8.0.0.0, **1**) reduced cell viability significantly at 3.12-50 µMby -13.2 % (3.12 µM; p<0.05), -15.0 % (6.25 µM; p<0.01), -16.5 % (12.5 µM; p<0.01), -26.3 % (25 µM; p<0.001) and -38.0 % (50 µM; p<0.001)compared to DMSO (Fig. 6).

In contrast, derivative NL_8.1.0.0 (**2**) at 25 µM caused a significant ($p<0.01$) increase of viability by +14.0 % ($p<0.01$)compared to DMSO (Fig. 6).NL_8.1.^{1}3.0 (**3a**) significantly diminished viability of GOS-3 cellsin a concentration-dependent manner by -26.0 % (6.25 µM, $p<0.01$), -61.5 % (12.5 µM, $p<0.01$), -97.0 % (25 µM, $p<0.001$) and -100 % (50 µM, $p<0.001$) compared to DMSO (Fig. 6). Moreover, NL_8.1.^{1}3.0 (**3a**) significantly inhibited the viability by -41.4 % (12.5 µM, $p<0.001$), -70.6 % (25 µM, $p<0.001$) and -71.6 % (50 µM, $p<0.01$) compared to 5-FUrd(Fig. 6).

However, in contrast to NL_8.1.^{1}3.0 (**3a**), derivative NL_8.1.^{1}3.0*P*(**4a**) (1.56-50 µM) significantly raised cell viability at all concentrations by +26.8 % (1.56 µM; $p<0.001$), +29.7 % (3.12 µM; $p<0.001$), +32.7 % (6.25µM; $p<0.001$), +22.4 % (12.5 µM; $p<0.01$), +31.2 % (25µM; $p<0.001$) and + 16.4 % (50 µM; $p<0.001$) compared to DMSO (Fig. 6).

Substance NL_8.1.^{1}1.0*P*(**4b**)revealedno significant effects on cellviability of GOS-3 cells (Fig. 6).

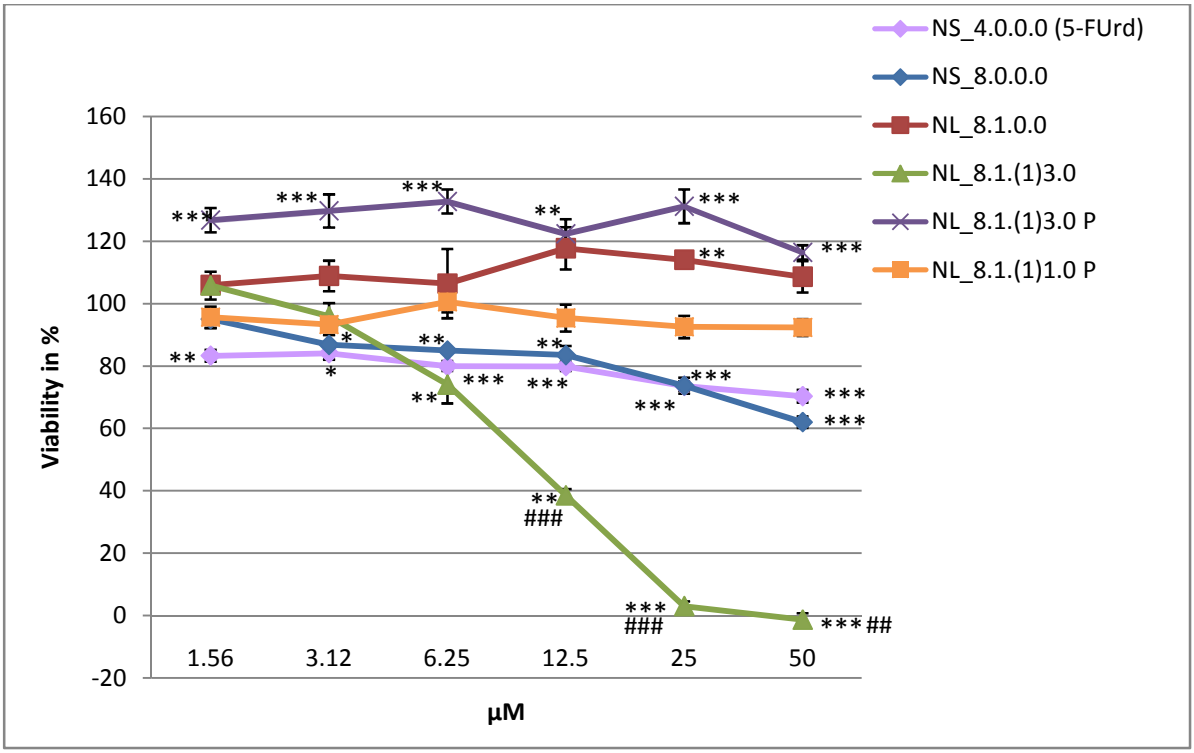


Figure 6.Viability (in %) of human GOS-3 glioblastoma cells after incubation (48h) with NS_4.0.0.0 (5-FUrd), formycin A (NS_8.0.0.0, **1**) or its derivatives (NL_8.1.0.0,**2**, NL_8.1.^{1}3.0,**3a**, NL_8.1.^{1}3.0 *P*, **4a**, and NL_8.1.^{1}1.0*P,* **4b**) is shown. Values are given (in % viability of control [incubation with

medium alone; = 100 % viability]) as mean ± SEM; *p<0.05, **p<0.01, ***p<0.001, significance vs. DMSO control (equal concentration of DMSO to 50 µM substance solution), #p<0.05, ##p<0.01, ###p<0.001, significance vs. 5-FUrd at equal concentration; n=5 independent experiments assayed in quadruplicates.

Formycin B (NS_9.0.0.0, **5**) increased the cellviability of GOS-3 cells at 1.56, 3.12, 6.25 and 12.5 µMby +26.1 % (p<0.001), +16.8 % (p<0.05), +15.3 % (p<0.05) and +19.4 % (p<0.05), respectively,compared to DMSO (Fig. 7).Derivative NL_9.1.^{1}3^{6}3.0(**7**) only at a concentration of 1.56 µM led to a significant (p<0.01) increase of the cell viability by +18.0%compared to DMSO (Fig.7).Also NL_9.1.0.0 (**6**) at a concentration of 1.56 µM increased the viability of GOS-3 cellssignificantly (p<0.01) by +16.9 %, but at the concentration of 50 µM it significantly (p<0.05) reduced cell viability by -6.4 % (Fig.7).

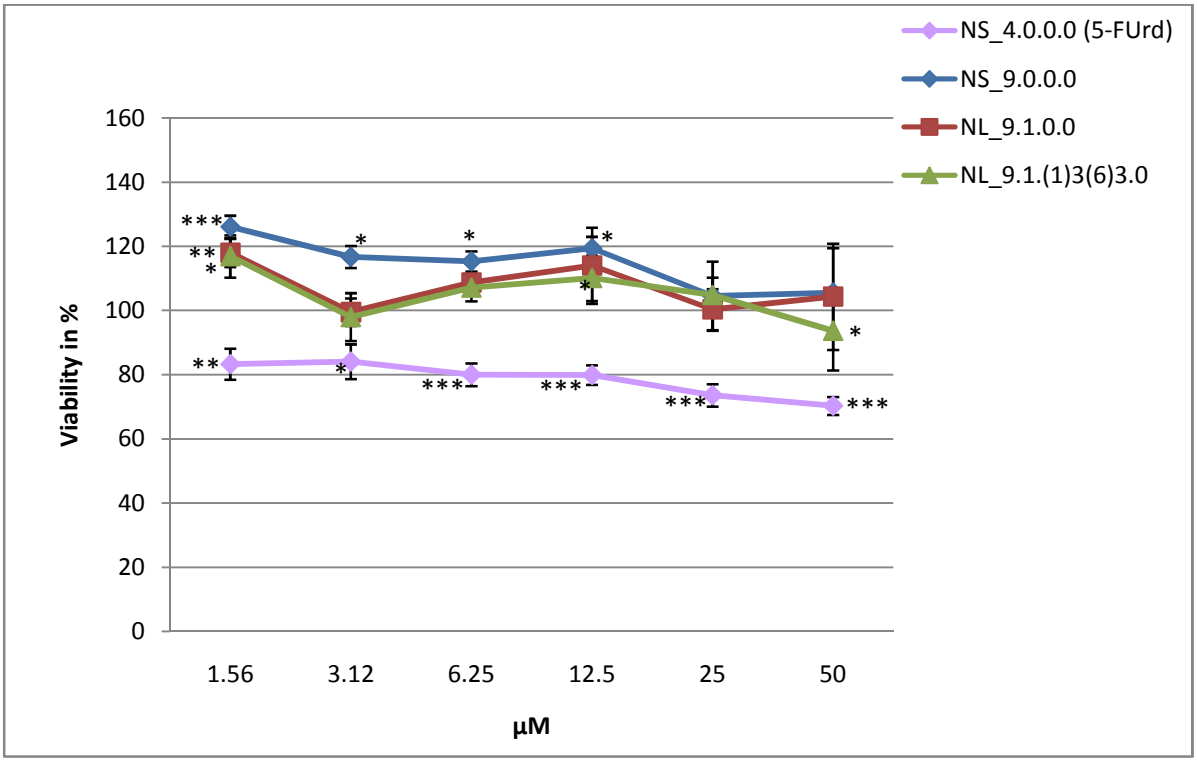


Figure 7.Viability (in %) of human GOS-3 glioblastoma cells after incubation (48h) with NS_4.0.0.0 (5-FUrd), Formycin B (NS_9.0.0.0, **5**) or its derivatives (NL_9.1.0.0, **6**, and NL_9.1.^{1}3^{6}3.0, **7**) is shown. Values are given (in % viability of control [incubation with medium alone; = 100 % viability]) as mean ± SEM; *p<0.05, **p<0.01, ***p<0.001, significance vs. DMSO control (equal concentration of DMSO to 50 µM substance solution), n=5 independent experiments assayed in quadruplicates.

After incubation (48h) of human U-87MG glioblastoma cells with NS_4.0.0.0 (5-FUrd), we observed a significant inhibition of cellviability (Figs. 8, 9). At all concentrations tested, 5-FUrd significantly ($p<0.001$) reduced viabilityby -68.8 % (1.56 µM), -71.6 % (3.12 µM), -67.8 % (6.25 µM), -79.7 % (12.5 µM), -71.1 % (25 µM) and -69.6 %(50 µM) compared to DMSO (~100% viability) (Figs. 8, 9).

Formycin A (NS_8.0.0.0, **1**)significantly reduced cell viability by -17.2 % (1.56 µM, $p<0.01$), -47.9 % (3.12 µM, $p<0.001$), -70.6 % (6.25 µM, $p<0.001$), -84.2 % (12.5 µM, $p<0.01$), -93.3 % (25 µM, $p<0.001$) and -97.1 % (50 µM, $p<0.001$)compared to DMSO (Fig. 8).Interestingly, NS_8.0.0.0(**1**) significantly ($p<0.001$) reduced the viability of U-87 MG cells by -11.6 % (12.5 µM), -20.2 % (25 µM) and -26.2 % (50 µM) in comparison to 5-FUrd (Fig. 8).

Moreover, at all concentrations tested, derivative NL_8.1.^{1}3.0(**3a**) significantly inhibitedcell viability by -23.0 % (1.56 µM; $p<0.001$), -39.1 % (3.12 µM; $p<0.001$), -51.1 % (6.25 µM; $p<0.001$), -55.3 % (12.5 µM; $p<0.001$), - 86.2 % (25 µM; $p<0.01$) and -90.2 % (50 µM; $p<0.01$)compared to DMSO(Fig. 8). Interestingly, NL_8.1.^{1}3.0(**3a**) significantlydiminished the cell viability by -15.1 % (25 µM, $p<0.01$) and -20.7 % (50 µM, $p<0.05$) compared to 5-FUrd (Fig. 8).

NL_8.1.^{1}1.0*P*(**4b**) showed only at 3.12 µMa significant ($p<0.01$) inhibition of cell viability by -6.0 % compared to DMSO (Fig. 8).In contrast, compounds NL_8.1.0.0 (**2**), and NL_8.1.^{1}3.0*P*(**4a**)revealedno significant effects on viability of U-87 MG cells compared to DMSO (Fig. 8).

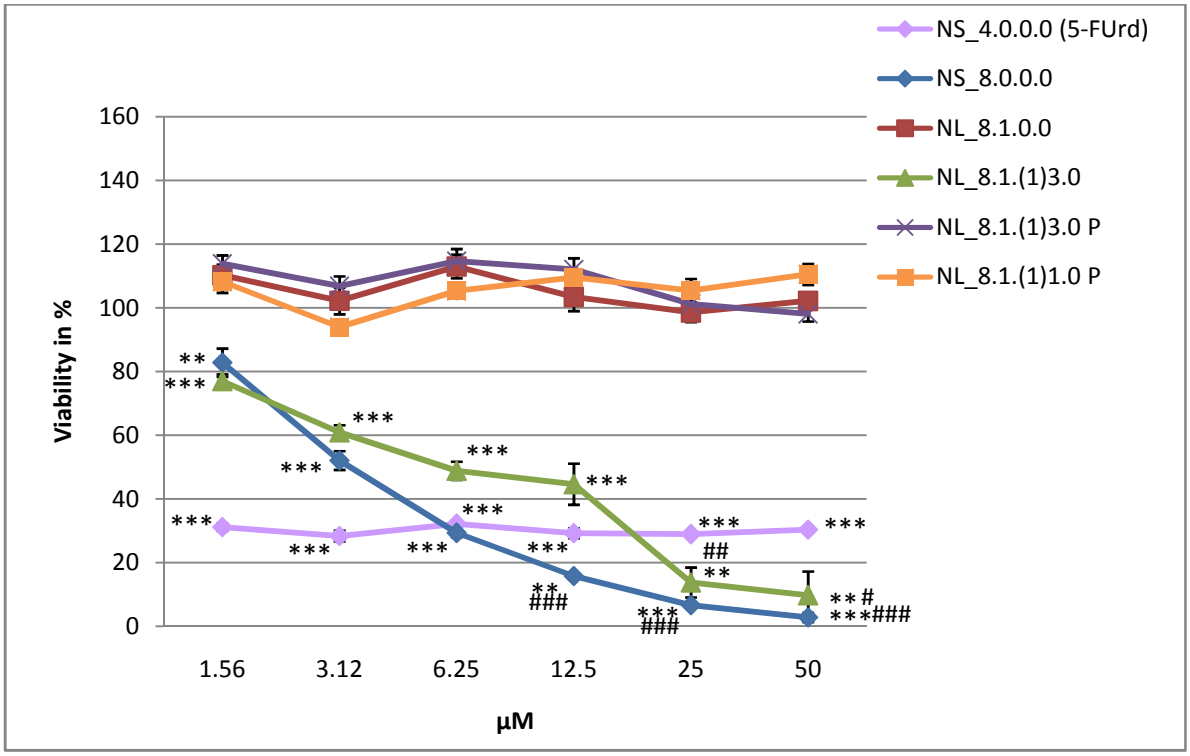


Figure 8.Viability (in %) of human U-87 MG glioblastoma cells after incubation (48h) with NS_4.0.0.0 (5-FUrd), Formycin A (NS_8.0.0.0, **1**) or its derivatives (NL_8.1.0.0,**2**, NL_8.1.^{1}3.0, **3a**, NL_8.1.^{1}3.0*P*(**4a**) and NL_8.1.^{1}1.0*P,* **4b**) is shown. Values are given (in % viability of control [incubation with medium alone; = 100 % viability]) as mean ± SEM; *p<0.05, **p<0.01, ***p<0.001, significance vs. DMSO control (equal concentration of DMSO to 50 µM substance solution), $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001, significance vs. 5-FUrd at equal concentration; n=5 independent experiments assayed in quadruplicates.

At concentrations of 25 and 50 µM,Formycin B (NL_9.0.0.0, **5**) significantly (p<0.001) reduced viability of U-87 MG cells by -24.5 % and -37.5 % compared to DMSO (Fig. 9).At concentrations of 12.5, 25 and 50 µM NL_9.1.^{1}3^{6}3.0 (**7**),a significant (p<0.001) inhibition of viability of U-87 MG cellsby -11.2 %, -69.5 % and -99.2 % in comparison to DMSO (Fig. 9) was observed. Additionally,NL_9.1.^{1}3^{6}3.0(**7**) significantly (p<0.001) decreased viabilityof human U-87 MG glioblastoma cellsby -28.3 % at 50 µMcompared to 5-FUrd (Fig. 9) whereas NL_9.1.0.0 (**6**) showed no significant effects on the viability of human U-87 MG glioblastoma cells compared to DMSO (Fig. 9).

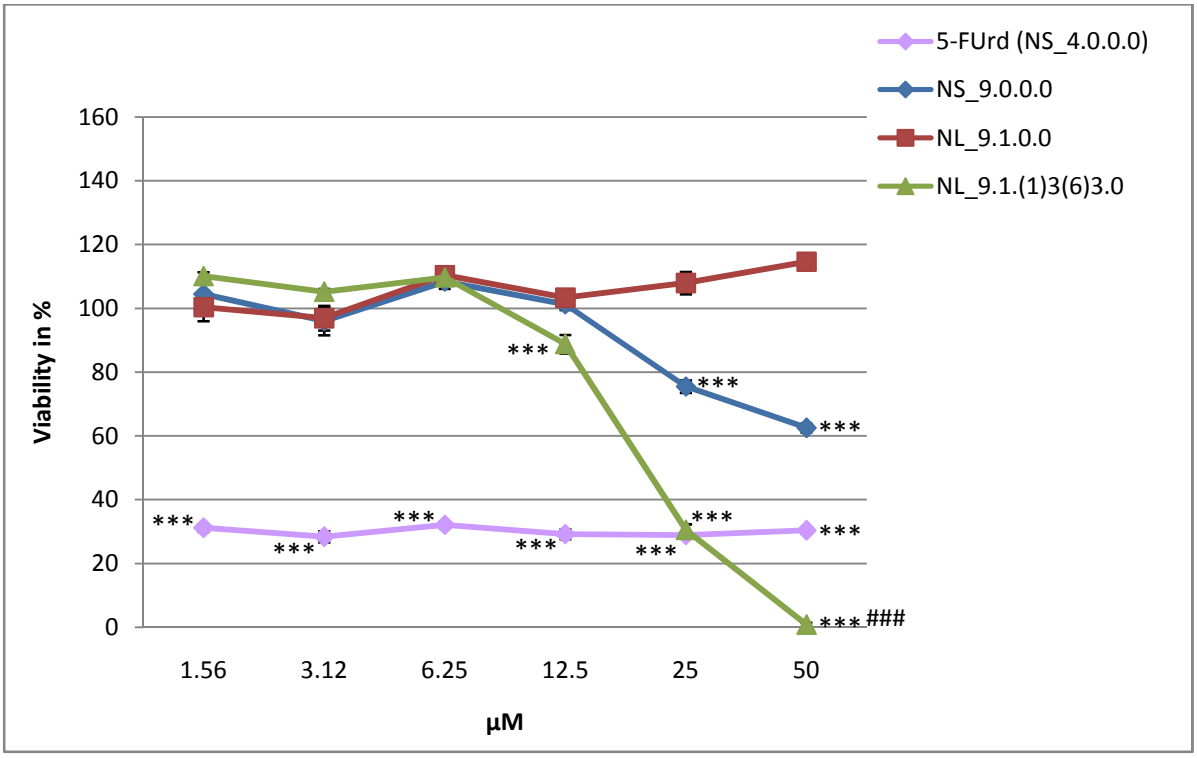


Figure 9.Viability (in %) of human U-87 MG glioblastoma cells after incubation (48h) with NS_4.0.0.0 (5-FUrd), Formycin B (NS_9.0.0.0, **5**) or its derivatives (NL_9.1.0.0, **6**and NL_9.1.^{1}3^{6}3.0, **7**) is shown. Values are given (in % viability of control [incubation with medium alone; = 100 % viability]) as mean ± SEM; *p<0.05, **p<0.01, ***p<0.001, significance vs. DMSO control (equal concentration of DMSO to 50 µM substance solution), n=5 independent experiments assayed in quadruplicates.

Next, we used a primary Glioblastoma patient-derived cell line GBM 2014/42 for cell viability assays. After treatment (48h) of GBM 2014/42cellswith NS_4.0.0.0 (5-FUrd), we observed that5-FUrd significantly (p<0.001) reduced the viabilityat alltested concentrations by -71.3 % (1.56 µM), -74.1 % (3.12 µM), -71.1 % (6.25 µM), -72.7 % (12.5 µM), 73.2 % (25 µM) and -70.9 % (50 µM) in comparison to DMSO (~100% viability) (Figs. 10, 11).

Furthermore, Formycin A (NS_8.0.0.0, **1**) significantly (p<0.001) decreased viability of GBM 2014/42 cellsat all concentrationstestedby -58.2 % (1.56 µM), -80.0 % (3.12 µM), -92.3 % (6.25 µM), -95.3 % (12.5 µM), -100 % (25 µM) and -99.6 % (50 µM)compared to DMSO (Fig. 10).Additionally, NS_8.0.0.0 (**1**) significantlyreducedthe viability by -5.9 %

(3.12 µM, p<0.05), -21.1 % (6.25 µM, p<0.001), -22.7 % (12.5 µM, p<0.001), -29.9% (25 µM, p<0.001) and -28.6 % (50 µM, p<0.001) in comparison to 5-FUrd (Fig. 10).

At all concentrations tested,NL_8.1.^{1}3.0(**3a**) significantly diminished the viability of human primary glioblastoma cells GBM 2014/42 by -20.8 % (1.56 µM, p<0.001), -55.4 % (3.12 µM, p<0.01), - 65.4 % (6.25 µM, p<0.01), -80.3 % (12.5 µM, p<0.001), -100 % (25 µM, p<0.001) and -100 % (50 µM, p<0.001) in comparison to the DMSO control (Fig. 10). Moreover, NL_8.1.^{1}3.0 significantly inhibited the viability by -7.6 % (12.5 µM, p<0.01), -40.9 % (25 µM, p<0.001) and -39.5 % (50 µM, p<0.001) compared to 5-FUrd (Fig. 10).

Only at a concentration of 50µM,NL_8.1.^{1}3.0*P*(**4a**)caused a significant reduction in viability of GBM 2014/42 cellsby -18.4 % (p<0.01) compared to DMSO (Fig. 10). Compounds NL_8.1.0.0 (**2**), and NL_8.1.^{1}1.0*P*(**4b**)revealedno significant effects on the viability of GBM 2014/42cells compared to DMSO (Fig. 10).

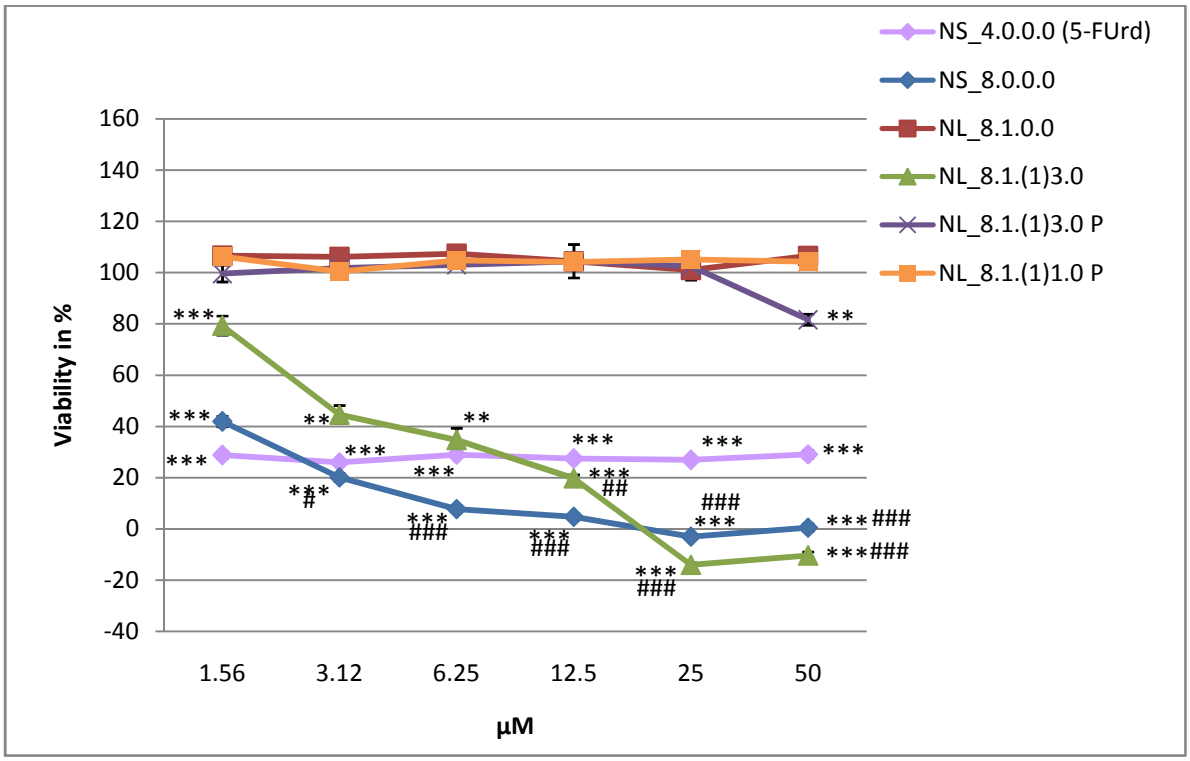


Figure 10.Viability (in %) of human primary glioblastoma cells (GBM 2014/42) after incubation (48h) with NS_4.0.0.0 (5-FUrd), Formycin A (NS_8.0.0.0, **1**) or its derivatives (NL_8.1.0.0, **2**, NL_8.1.^{1}3.0,**3a**, NL_8.1.^{1}3.0*P*, **4a,** and NL_8.1.^{1}1.0*P*, **4b**) is shown. Values are given (in % viability

of control [incubation with medium alone; = 100 % viability]) as mean ± SEM; *p<0.05, **p<0.01, ***p<0.001, significance vs. DMSO control (equal concentration of DMSO to 50 µM substance solution), #p<0.05, ##p<0.01, ###p<0.001, significance vs. 5-FUrd at equal concentration; n=5 independent experiments assayed in quadruplicates.

At concentrations of 12.5, 25 and 50 µM,Formycin B (NL_9.0.0.0, **5**) significantly (p<0.001) inhibited the viability of GBM 2014/42 cells by -15.4 %, -48.4 % and -59.2 % compared to DMSO (Fig. 11).Only at a concentration of 50 µM,the derivative NL_9.1.^{1}3^{6}3.0 (**7**)led to a significant (p<0.01) reduction of cell viability in GBM 2014/42 cellsby -87.6% compared to DMSO (Fig. 11). Additionally,NL_9.1.^{1}3^{6}3.0 (**7**)significantly (p<0.001) diminished the viability of by -16.3 % (50 µM) in comparison to 5-FUrd(Fig. 11). NL_9.1.0.0 (**6**) showedno significant effects on cellviability compared to DMSO (Fig. 11).

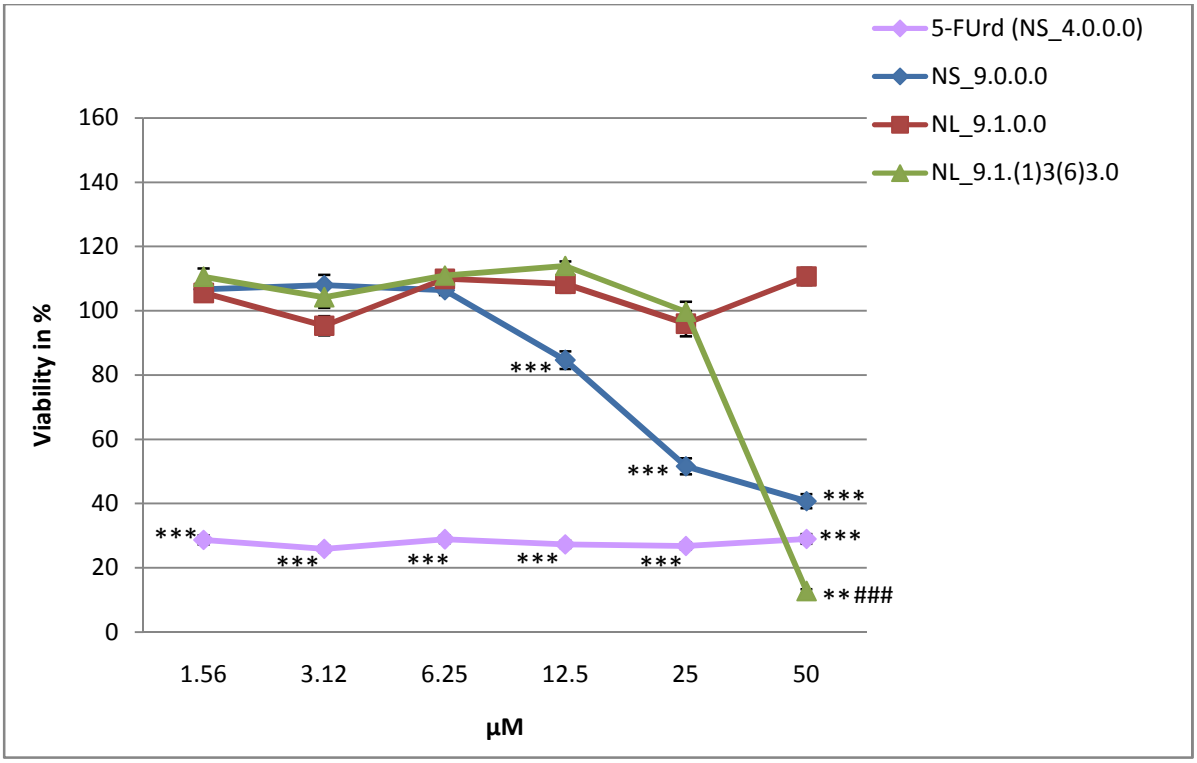


Figure 11.Viability (in %) of human primary glioblastoma cells (GBM 2014/42) after incubation (48h) with NS_4.0.0.0 (5-FUrd), Formycin B (NS_9.0.0.0, **5**) or its derivatives (NL_9.1.0.0, **6**, and NL_9.1.^{1}3^{6}3.0, **7**) is shown. Values are given (in % viability of control [incubation with medium alone; = 100 % viability]) as mean ± SEM; *p<0.05, **p<0.01, ***p<0.001, significance vs. DMSO control (equal concentration of DMSO to 50 µM substance solution), n=5 independent experiments assayed in quadruplicates.

Moreover, the higher concentrations (12.5, 25 and 50 µM) of the most effective formycin A derivative NL_8.1.[1]3.0 were auxiliary tested on BT4Ca, GOS-3 and U-87 MG by using a second viability/cytotoxicity assay (sulforhodamin B assay), indeed confirming the cytotoxic effects of Formycin A derivative NL_8.1.[1]3.0 (data not shown).

In addition to the significant cytotoxic effects of Formycin A derivative NL_8.1.[1]3.0; we found that this substance is also cytotoxic (at various concentrations; after incubation for 48h) for other tumor cell lines, i.e. human colorectal carcinoma (HT29), human hepatocellular carcinoma (HEPG2), human pancreas carcinoma cells (Panc-1) and murine renal carcinoma (RenCa) cells (data not shown).

As previously published concerning other anti-tumor derivatives (Knies et al., 2015a, 2015b, 2016; Hammerbacher et al. 2018), the novel derivatives were tested on PMA-differentiated human THP-1 macrophages to estimate potential (cytotoxic) side effects on immune cells.Incubation (48h) of PMA-differentiated human THP-1 macrophageswith NS_4.0.0.0 (5-FUrd) revealed a marginal inhibition of cell viability(Figs.12, 13). At all concentrations tested, 5-FUrd significantly reduced the viabilityof PMA-differentiated human THP-1 macrophages by -0.8 % ($p<0.05$), -2.7 % ($p<0.01$), -5.2 % ($p<0.01$), -5.0 % ($p<0.01$), -6.1 % ($p<0.001$) and -6.8 %($p<0.01$) in comparison to DMSO (~100% viability) (Figs. 13, 14).Unlike, Formycin A (NS_8.0.0.0, **1**) significantly diminished cell viability of THP-1 macrophagesat alltested concentrations by -11.5 % (1.56 µM, $p<0.001$), -19.4 % (3.12 µM, $p<0.01$), -28.1 % (6.25 µM, $p<0.01$), -43.0 % (12.5 µM, $p<0.01$), -50.5 % (25 µM, $p<0.001$) and -64.4 % ($p<0.001$)compared to DMSO (Fig. 12).

Derivative NL_8.1.[1]3.0(**3a**) decreased the viability of PMA-differentiated human THP-1 macrophages by -26.0 % (6.25 µM, $p<0.01$), -61.5 % (12.5 µM, $p<0.01$), -97.0 % (25 µM, $p<0.001$) and -100 % (50 µM, $p<0.001$) compared to the DMSO control (Fig.

7).Moreover, NL_8.1.^{1}3.0(**3a**) significantlyinhibited the viability of PMA-differentiated human THP-1 macrophagesby -41.4 % (12.5 µM, p<0.001), -70.6 % (25 µM, p<0.001) and -71.6 % (50 µM, p<0.01) in comparison to 5-FUrd (Fig. 12)

At concentrations of 6.25, 12.5, 25 and 50 µM, NL_8.1.^{1}3.0(**3a**) led to a significant reduction of viability in human THP-1 macrophagesby -5.5 % (p<0.01), -57.9 % (p<0.01), -89.2 % (p<0.001) and -99.6 % (p<0.001) compared to the DMSO control (Fig. 12). Additionally, NL_8.1.^{1}3.0(**3a**) significantly reduced the viability of PMA-differentiated human THP-1 macrophagesby -52.9 (12.5 µM, p<0.01), -83.1 % (25 µM, p<0.001) and -92.8 % (50 µM, p<0.01) compared to 5-FUrd (Fig. 12).

In comparison with derivative NL_8.1.^{1}3.0 (**3a**), at all tested concentrations, NL_8.1.^{1}3.0*P* (**4a**) showed a proliferative effect, i.e.significantly increased the viability of human THP-1 macrophages by +13.2 % (1.56 µM; p<0.05), +14.0 % (3.12 µM; p<0.01), +23.5 % (6.25 µM; p<0.05), +26.9 % (12.5µM; p<0.05), +43.3 % (25µM; p<0.01) and + 47.4 % (50µM; p<0.001) compared to DMSO (Fig. 12).The compoundsNL_8.1.0.0 (**2**), and NL_8.1.^{1}1.0*P*(**4b**) revealedno significant effects on the viability of PMA-differentiated human THP-1 macrophages in comparison to DMSO (Fig. 12).

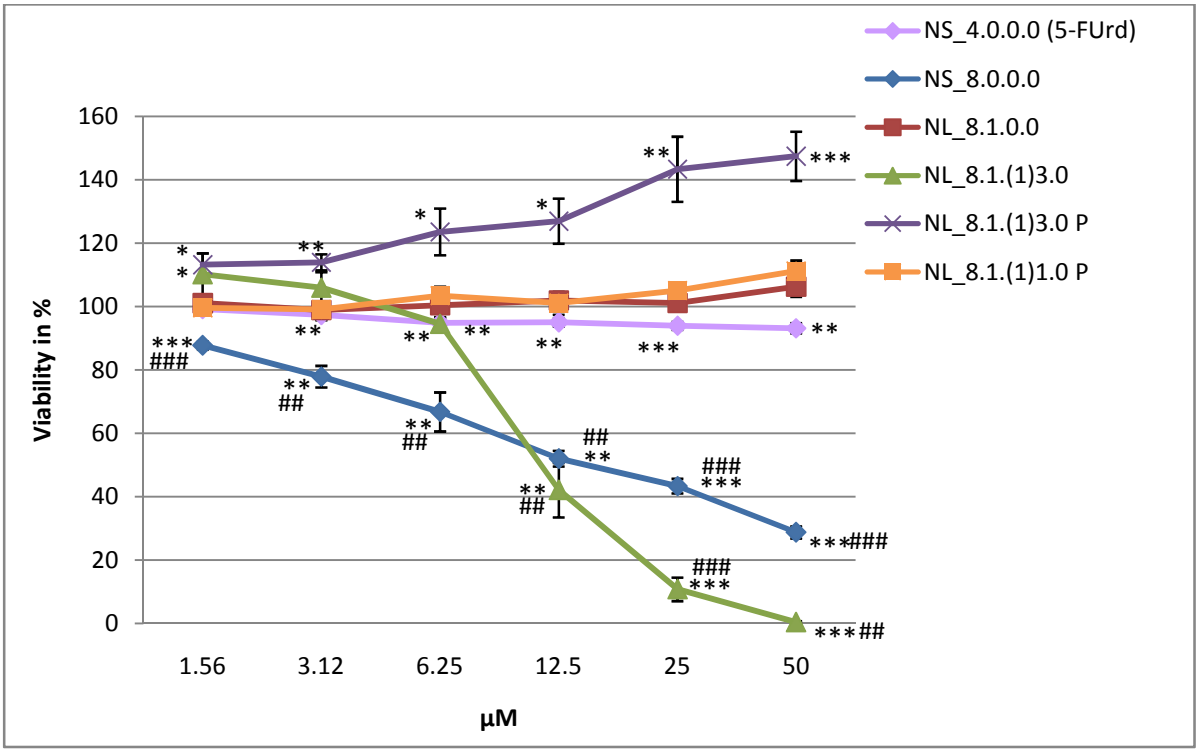


Figure 12. Viability (in %) of PMA-differentiated human THP-1 macrophages after incubation (48h) with NS_4.0.0.0 (5-FUrd), Formycin A (NS_8.0.0.0, **1**) or its derivatives (NL_8.1.0.0,**2**, NL_8.1.^{1}3.0,**3a,** NL_8.1.^{1}3.0*P,* **4a**, and NL_8.1.^{1}1.0*P*, **4b**) is shown. Values are given (in % viability of control [incubation with medium alone; = 100 % viability]) as mean ± SEM; *p<0.05, **p<0.01, ***p<0.001, significance vs. DMSO control (equal concentration of DMSO to 50 µM substance solution), $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001, significance vs. 5-FUrd at equal concentration; n=5 independent experiments assayed in quadruplicates.

Only at a concentration of 50 µM,Formycin B (NL_9.0.0.0, **5**) significantly (p<0.05) diminished the viability human THP-1 macrophages by -19.8 % compared to the DMSO control (Fig. 13). NL_9.1.0.0 (**6**) and NL_9.1.^{1}3^{6}3.0(**7**)revealed no significant effects on the viability of PMA-differentiated human THP-1 macrophages compared to DMSO(Fig. 13).

10.1002/cbdv.201900012

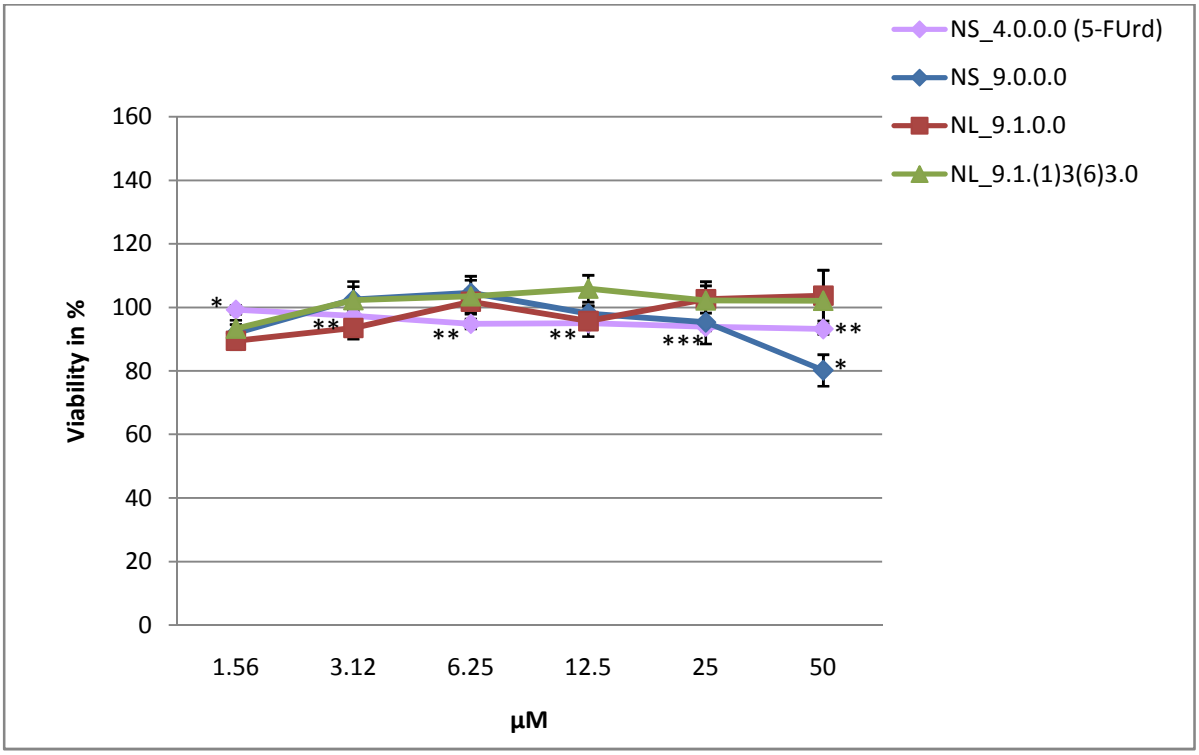


Figure 13. Viability (in %) of PMA-differentiated human THP-1 macrophages after incubation (48h) with NS_4.0.0.0 (5-FUrd), Formycin B (NS_9.0.0.0, **5**) or its derivatives (NL_9.1.0.0, **6**, and NL_9.1.^{1}3^{6}3.0, **7**) is shown. Values are given (in % viability of control [incubation with medium alone; = 100 % viability]) as mean ± SEM; *p<0.05, **p<0.01, ***p<0.001, significance vs. DMSO control (equal concentration of DMSO to 50 µM substance solution); n=5 independent experiments assayed in quadruplicates.

Because Formycin Aderivative NL_8.1.^{1}3.0 inhibited the viability of glioblastoma cells, we have exemplarily analyzed a possible molecular mechanism to explain the cytotoxic effects, and we found that Formycin Aderivative NL_8.1.^{1}3.0 significantly increased the active caspase-3 activity in BT4Ca cells, which is interpreted as apoptosis induction (data not shown).

5. Experimental Part.

5.1. Nomenclature. For the general numbering of nucleosides and nucleolipids, a novel denomination system was developed, which allows an easy comparison of compound data among the various publications of our groups and which is disclosed in ref. [14]. Moreover, it was deposited sustainably in the repository of the library of the University of Osnabrück under the following unique registration number (URN) and URL:

https://repositorium.uni-osnabrueck.de/handle/urn:nbn:de:gbv:700-2015110413639.

Nucleolipids are abbreviated as“NL”, nucleosides are abbreviated as“NS”. The first number refers to the nucleosides. The second number refers to the moiety at the 2’,3’-position at the glyconic ring; cyclic moieties are abbreviated by “cycl” before the number. The third number refers to the lipophilic moiety at the base [N(3) for pyrimidines, N(1) for purines]. The forth number refers to a lipophilic moiety at the 5’-O position. Identical residues carry the same number; “0” stands for a molecule without a residue at this position. For a translation of the NS/NL nomenclature to the plain compound numbers used throughout the text, schemes and figures see Table 2.

Table 2. Translation of plain compound numbers (Schemes 1 and 2) into the NS/NL nomenclature.

1	**NS_8.0.0.0**
2	**NL_8.1.0.0**
3a	**NL_8.1.^{1}1.0**
3b	**NL_8.1.^{1}3.0**
4a	**NL_8.1.^{1}1.0*P***
4b	**NL_8.1.^{1}3.0*P***
5	**NS_9.0.0.0**
6	**NL_9.1.0.0**
7	**NL_9.1.^{1}3.^{6}3.0**

5.2. Chemistry.

5.2.1.**General Remarks**. All chemicals were purchased from Sigma-Aldrich (DE-Deisenhofen) or TCI Europe (BE-Zwijnrecht). Formycin A was a generous gift of Mr. *Klaus Mühlegger, Roche Diagnostics DE-Penzberg*). Solvents were laboratory grade and were distilled before use. Column chromatography was performed on silica gel 60 (CAS 112926-00-8). Thin layer chromatography (TLC) was performed using aluminum sheets and silica gel 60 F_{254}; 0.2 mm layer (Merck, Germany). NMR spectra (^{1}H, ^{13}C, DEPT-135, gated-decoupled ^{13}C) were obtained using an AMX-500 instrument (Bruker, DE-*Rheinstetten*, Germany): ^{1}H: 500.14 MHz, ^{13}C: 125.76 MHz; chemical shifts (δ) are reported in ppm referenced to an internal standard of residual proteosolvent [D_6]DMSO (2.50. 39.50 ppm, relative to tetramethylsilane (TMS) as internal standard). Multiplicity is quoted as br (broad), s (singlet), d (doublet), t (triplet), q (quartet), quint (quintet), m (multiplet), *Ψ*t (pseudotriplet), dd (doublet of doublet), ddd (doublet of doublet of doublets). *J* values are reported in Hz. 2D [^{1}H,^{1}H] and [^{1}H,^{13}C] correlation spectra (heteronuclear single quantum coherence, HSQC) and Cosy Long Range spectra (pulse program: cosygpmfph) were measured with the same instrument. Sample preparation was performed as follows: an appropriate amount of compound (usually 20 – 25 mg) was dissolved in [D_6]DMSO (0.5 ml) and placed in the NMR quartz tube (diameter, 5 mm). Before measurement the solutions were degassed by ultrasonication for several minutes. Number of scans: ^{1}H: 64, ^{13}C: 12.000, DEPT-135: 5.000. ESI MS was performed by a Bruker Daltronics Esquire HCT instrument (Bruker daltronics, DE-Leipzig); ionization was performed with a 2% formic acid (HCOOH) solution in MeCN. UV/Vis spectra were obtained using a Cary 1E spectrophotometer (Varian, DE-Darmstadt). Compound samples of about 1mg were dissolved either in MeOH, or an appropriate buffer solution (pH 3, 7 or 9, 100 ml, each). Aliquots of the fully dissolved compounds (warming, ultrasonication) were subjected to UV/Vis spectrometry in MT4

quartz cuvettes (Hellma, DE-Darmstadt). Elemental analyses (C, H, N) were performed on a VarioMICRO instrument (Fa. Elementar, DE-Hanau). $^{10}\log P_{OW}$ values were determined *in silico* using the http://eadmet.com/de/physprop.phpwebsite with *ePhysChem* that contains ALOGPS v.3.0. [15,16]. Experimental determination of $^{10}\log P_{ChW}$ values of compounds were performed as follows: samples of compounds (2 mg, each) were dissolved in a heterogenic mixture of cyclohexane (25 ml) and water (25 ml) by ultrasonication (10 min) under slightly warming. After separation of the layers from each phase aliquots of 1 ml were withdrawn, and their UV spectra were run in 1cm quartz cuvettes. From the ratio of maximal extinctions of both layers at λ_{max} the corresponding $^{10}\log P_{ChW}$ values were calculated.

5.3. Syntheses.

*Formycin A (***1,NS_8.0.0.0***).* ^{1}H-NMR (500.13 MHz, DMSO-d_6):12.68 (*s*, br. NH); 8.15 (*s*, H-C(5)); 7.36 (*s*, br., NH_2); 5.85 (*s*, H-C(1‘)); 4.94-4.93 (*m*, HO-C(5’), HO-C(2’)); 4.87 (*d*, 1H, ^3J(HO-C(3’),H-C(3’)) = 3.5, HO-C(3’)); 4.52-4.49 (*m*, 1H, H-C(2’)); 4.11-4.09 (*m*, 1H, H-C(3’)); 3.93 (*ψq*, 1H, ^3J(H-C(4’),H_2-C(5’)) = 3.0, ^3J(H-C(4’), H-C(3’)) = 3.0, H-C(4’)); 3.67-3.64 (m, 2H, H_2-C(5’)).^{13}C-NMR (125.76 MHz, DMSO-d_6):151.26 (C(5)); 150.73 (C(7)); 144.04 (C(3)); 138.81 (C(3a)); 122.15 (C(7a)); 85.93 (C(4‘)); 78.40 (C(1‘)); 75.00 (C(2‘)); 72.36 (C(3‘)); 62.54 (C(5‘)).

3-((2S,3R,4S,5R)-3,4-Dihydroxy-5-(hydroxymethyl)-tetrahydrofuran-2-yl)-1H-pyrazolo[4,3-d]pyrimidin-7(6H)-one (**5**, **NS_9.0.0.0**; Formycin B) by enzymatic deamination: 0.8 g (3.75 mmol) of Formycin A (**1**, **NS_8.0.0.0**), suspended in ultrapure H_2O(27 ml), were charged dropwise with a solution of adenosine desaminase (0.75 ml, 90 units, calf intestine). The reaction mixture was stirred for 5 d at ambient temperature (TLC-control). The milky-white suspension formed was evaporated to dryness yielding a colorless powder of formycin B (0.75 g, 3.75 mmol, 100 %). TLC (SiO_2*60*,

CH_2Cl_2/MeOH,75:25; v/v)): R_f, 0.48. UV (MeOH):λ_{max} 278.3 nm (ε = 7.200 mol^{-1} cm^{-1}). log*P* (ALOGPS 3.01): -1.40 ± 0.74.^{1}H-NMR (500.13 MHz, DMSO-d_6): 7.87 (H-C(5)); 4.92 (*d*, 1H, ^3J(H-C(1'),H-C(2')) = 7.0, H-C(1')); 4.43 (*dd*, 1H, ^3J(H-C(2'),H-C(1')) = 6.5, ^3J(H-C(2'),H-C(3')) = 5.5, H-C(2')); 4.06 (*dd*, 1H, ^3J(H-C(3'),H-C(2')) = 5.0, ^3J(H-C(3'),H-C(4')) = 4.0, H-C(3')); 3.88 (*ψq*, 1H, ^3J(H-C(4'),H-C(3')) = 3.5, ^3J(H-C(4'),H_2-C(5')) = 3.5, H-C(4')); 3.63-3.46 (*m*, partially superimposed by HOD, H_2-C(5')). ^{13}C-NMR (125.76 MHz, DMSO-d_6): 153.86 (C(7)); 143.06 (C(5)); 142.87 (C(3)); 136.43 (C(3a)); 129.52 (C(7a)); 85.55 (C(4')); 77.35 (C(1')); 74.76 (C(2')); 71.99 (C(3')); 62.46 (C(5')). ESI-MS: 269.04 $[M+H]^+$, 537.18 $[2M+H]^+$; calculated: 268.23.

Ethyl3-[4-(7-amino-1H-pyrazolo[4,3-d]pyrimidin-3-yl)-6-hydroxymethyl-2-methyl-tetrahydro-furo[3,4-d][1,3] dioxol-2-yl]-propionate(**2**, **NL_8.1.0.0**). Anhydrous formycin A(**1**, **NS_8.0.0.0**; 0.75 g; 2.805 mmol) was dissolved in dry dimethylformamide (12 ml). Subsequently, ethyl levulinate (0.75 ml; 5.332 mmol), triethyl orthoformate (0.69 ml; 4.211 mmol) and 4 M HCl in 1.4-dioxane (2.55 ml) were added. After stirring for 24 h at room temperature the reaction mixture was partitioned betweenCH_2Cl_2 (120 ml) and a saturated aqueous $NHCO_3$ solution (35 ml). The aqueous layer was back-extracted thrice (30, 20 and 15 ml CH_2Cl_2). The combined organic layers were evaporated on a rotary evaporator and then co-evaporated repeatedly from CH_2Cl_2 to remove residual DMF. The residue was dried overnight in high *vacuo* at 40 °C. Purification of the title compound was performed by silica gel chromatography(SiO_2*60*, column: 6.5 x 11 cm; CH_2Cl_2/MeOH 85:15; v/v). After pooling of appropriate fractions and evaporation of the solvent the product was isolated as a colorless foam (0.705 g, 1.792 mmol, 64 %; diastereoisomeric mixture, [1*R*]/[1*S*]= 8.6:1). TLC (SiO_2*60,*CH_2Cl_2/MeOH 85:15; v/v):R_f, 0.71. UV (MeOH):λ_{max} 293 nm (ε = 11.150 mol^{-1} cm^{-1}), λ_{max} 304.5 nm (ε= 7.400 mol^{-1} cm^{-1}). logP_{OW}(ALOGPS 3.01): 0.18 ± 0.74. ^{1}H-NMR (500.13 MHz, DMSO-d_6),12.85 (*s*, br., 1H, NH); 8.19 (*s*, H-C(5)); 7.36 (*s*, br., 2H, NH_2);

5.40 (*s*, br., 1H, HO-C(5')); 5.28 (*s*, br., H-C(1')); 5.16-5.16 (*m*, 1H, H-C(2'), (1*R*)+(1*S*)); 4.88 (*dd*, 1H, ^3J(H-C(3'),H-C(4')) = 3.0, ^3J(H-C(3'),H-C(2')) = 7.0, H-C(3')(1*R*)); 4.85 (*dd*, 1H, ^3J(H-C(3'),H-C(4')) = 3.0, ^3J(H-C(3'),H-C(2')) = 7.0, H-C(3')(1*S*)); 4.14 (*ψq*, 1H, ^3J(H-C(4'),H-C(3')) = 4.0, ^3J(H-C(4'), H_2-C(5')) = 4.0, H-C(4')(1*S*)); 4.10 (*ψq*, 1H, ^3J(H-C(4'),H-C(3')) = 4.0, ^3J(H-C(4'), H2-C(5')) = 4.0, H-C(4')(1*R*)); 4.07 (*q*, 2H, ^3J(CH_2(ester),CH_3(ester)) = 7.0, CH_2(ester)(1*R*)); 4.01 (*q*, 2H, ^3J(CH_2(ester),CH_3(ester)) = 7.0, CH_2(ester)(1*S*)); 3.54-3.48 (*m*, 2H, H_2-C(5') (1*R*)+(1*S*)); 2.79 (*t*, 2H, ^3J(CH_2(C=O),CH_2(acetal)) = 7.3, CH_2(C=O)(1*R*)); 2.30 (*t*, 2H, ^3J(CH_2(C=O),CH_2(acetal)) = 7.3, CH_2(C=O)(1*S*)); 2.69 (*t*, 2H, ^3J(CH_2(acetal),CH_2(C=O)) = 7.3, CH_2(acetal)(1*R*)); 1.81(*t*, 2H, ^3J(CH_2(acetal),CH_2(C=O)) = 7.3, CH_2(acetal)(1*S*)); 1.43 (*s*, 3H, CH_3(acetal)(1*S*)); 1.23 (*s*, 3H, CH_3(acetal)(1*R*)); 1.10 (*t*, 3H, ^3J(CH_3(ester),CH_2(ester)) = 7.0, CH_3(ester)(1*R*)); 1.13 (*t*, 3H, ^3J(CH_3(ester),CH_2(ester)) = 7.0, CH_3(ester)(1*S*)).^{13}C-NMR (125.76 MHz, DMSO-d_6),172.64 (C=O(ester)(1*R*)); 172.53 (C=O(ester)(1*S*)); 151.89 (C(5)); 150.75 (C(7)); 142.65 (C(3)); 139.15 (C(3a)); 122.17 (C(7a)); 114.12 (C(acetal)(1*S*)); 113.99 (C(acetal)(1*R*)); 85.36 (C(4')(1*S*)); 85.02 (C(4')(1*R*)); 84.06 (C(1')(1*S*)); 83.30 (C(1')(1*R*)); 83.07 (C(3')(1*S*)); 82.39 (C(3')(1*R*)); 80.30 (C(2')(1*S*)); 79.69 (C(2')(1*R*)); 61.00 (C(5')(1*S*)); 61.87 (C(5')(1*R*)); 59.87 (CH_2(ester)(1*R*)); 59.82 (CH_2(ester)(1*S*)); 33.74 (CH_2(C=O)(1*R*)); 33.57 (CH_2(C=O)(1*S*)); 29.11 (CH_2(acetal)(1*S*)); 28.35 (CH_2(acetal)(1*R*)); 25.32 (CH_3(acetal)(1*S*)); 23.70 (CH_3(acetal)(1*R*)); 14.03 (CH_3(ester)(1*R*)); 13.96 (CH_3(ester)(1*S*)).ESI-MS: 394.16 $[M+H]^+$, 787.10 $[2M+H]^+$; calculated:393.39. Anal. Calc. for $C_{17}H_{23}N_5O_6$ (393.39): C, 51.90; H, 5.89; N, 17.80. Found: C, 51.87; H, 5.79; N,18.01.

Ethyl 3-((2S,3aS,4S,6R,6aR)-4-(7-amino-1-((2E,6E)-3,7,11-trimethyldodeca-2,6,10-trien-1-yl)-1H-pyrazolo[4,3-d]pyrimidin-3-yl)-6-(hydroxymethyl)-2-methyltetra hydrofuro[3,4-d][1,3]dioxol-2-yl)propanoate (**3b**, **NL_8.1.^{1}3.0**) and *Ethyl 3-((2S,3aR,4R,6S,6aS)-4-(hydroxymethyl)-2-methyl -6-(9-methyl-1-((2E,6E)-3,7,11-trimethyldodeca-2,6,10-trien-1-*

yl)-1,7-dihydropyrazolo[3,4-e] pyrimido[1,2-c] pyrimidin-3-yl)tetrahydrofuro[3,4-d] [1,3]dioxol-2-yl) propanoate(**4b**, **NL_8.1.^{1}3.0*P***).Anhydrous **NL_8.1.0.0** (**2**, 0.20 g; 0.534 mmol) was dissolved in dry and amine-free dimethylformamide (6 ml). After addition of dry K_2CO_3 (0.193 g; 1.4 mmol) and stirring of the suspension for 30 min at room temperature, *all-trans*farnesyl bromide (0.18 ml; 0.6 mmol) was added dropwise under N_2 atmosphere. Stirring was continued for 24 h. The salt was filtered off and washed three times. The filtrate and washings were evaporated to give a yellowish oil which was again co-evaporated from CH_2Cl_2 to remove residual DMF (water bath below 35 °C). The raw material was then further dried in high vacuo overnight. Separation ofthe products was performed by silica gel chromatography (SiO_2*60*, column: 5.5 x 17 cm; CH_2Cl_2/MeOH 95:5; v/v; 0.3 bar). The contents of two zones was isolated, and the solvent was evaporated. The faster migrating compound formed a colorless foam in high *vacuo*(**3b**, **NL_8.1.^{1}3.0**), the slower migrating compound formed a yellowish oil (**4b**, **NL_8.1.^{1}3.0*P***).

NL_8.1.^{1}3.0(**3b**): 0.120 g, 0.2 mmol, 37.4 %; diastereoisomeric mixture. TLC(SiO_2*60,* CH_2Cl_2/MeOH 95:5; v/v):R_f, 0.48. UV (MeOH):λ_{max} 305.8 nm (ε = 10.500 mol^{-1} cm^{-1}). logP_{OW}(ALOGPS 3.01): 3.61 ± 0.74. ^{1}H-NMR (500.13 MHz, DMSO-d_6), 8.16 (*s*, H-C(5)(1*R*+1*S*)); 7.29 (*s*, br, 2H, NH_2); 5.29-5.25 (*m*, 3H, H-C(10''), H-C(6''), HO-(5'), (1*R*+1*S*)); 5.18-5.17 (*m*, 2H, H-C(2'), H-C(2''), (1*R*+1*S*)); 5.08 (*d*, ^{3}J(H-C(1'),H-C(2')) = 4.5, H-C(1')(1*R*+1*S*)); 5.01 (*t*, ^{3}J(H_2-C(1''),H-C(2'')) = 6.5, H_2-C(1'')); 4.86 (*dd*, ^{3}J(H-C(3'),H-C(2')) = 7.0, ^{3}J(H-C(3'),H-C(4')) = 4.0, H-C(3')(1*R*+1*S*)); 4.09-4.05 (*m*, 3H, CH_2(ester)(1*R*+1*S*), H-C(4')(1*R*)); 4.02 (*ψq*, ^{3}J(CH_2(ester),CH_3(ester)) = 5.0, (CH_2(ester)(1*S*)); 3.54-3.42 (*m*, 2H, H_2-C(5')); 2.46 (*t*, ^{3}J(CH_2C=O,CH_2(acetal)) = 7.5, CH_2C=O(1*R*+1*S*)); 2.07-1.83 (*m*, 10H, H_2-C(4''), H_2-C(5''); H_2-C(8''); H_2-C(9''); CH_2(acetal), (1*R*+1*S*)); 1.77 (*s*, 3H, H_3-C(13'')(1*R*+1*S*)); 1.61 (s, 3H, H_3-C(14'')(1*R*+1*S*)); 1.52 (*s*, 3H, H_3-C(12'')(1*R*+1*S*)); 1.49 (*s*, 3H, H_3-C(15'')(1*R*+1*S*)); 1.29 (*s*, 3H, CH_3(acetal)(1*R*)); 1.20 (*t*,

^{3}J(CH_3(ester)(1*R*),CH_2(ester)(1*R*)) = 7.0, CH_3(ester)(1*R*)); 1.14 (*t*, ^{3}J(CH_3(ester)(1*S*),CH_2(ester)(1*S*)) = 7.0, CH_3(ester)(1*S*)).^{13}C-NMR (125.76 MHz, DMSO-d_6), 172.50 (C=O(ester)(1*R*)); 172.39 (C=O(ester)(1*S*)); 151.41 (C(5)(1*R*+1*S*)); 150.83 (C(7)(1*R*+1*S*)); 141.19 (C(3)(1*S*)); 141.08 (C(3)(1*R*)); 140.89 (C(3a)(1*R*+1*S*)); 139.35 (C(3'')(1*R*+1*S*)); 134.88 (C(7'')(1*R*+1*S*)); 130.44 (C(11'')(1*R*+1*S*)); 124.00 (C(6'')(1*R*+1*S*)); 123.28 (C(10'')(1*R*+1*S*)); 121.30 (C(7a)(1*R*+1*S*)); 119.62 (C(2'')(1*R*+1*S*)); 114.00 (C(acetal)(1*S*)); 113.93 (C(acetal)(1*R*)); 85.24 (C(1')(1*S*)); 84.89 (C(1')(1*R*)); 83.74 (C(4')(1*S*)); 82.99 (C(4')(1*R*)); 82.30 (C(2')(1*R*)); 79.97 (C(3')(1*S*)); 79.38 (C(3')(1*R*)); 61.90 (C(5')(1*S*)); 61.66 (C(5')(1*R*)); 59.75 (CH_2(ester)(1*S*)); 59.69 (CH_2(ester)(1*R*)); 38.99 (C(1'')(1*R*+1*S*)), 38.69 (C(4'')(1*R*+1*S*)); 38.38 (C(8'')(1*R*+1*S*)); 33.61 (CH_2C=O(1*R*)); 33.77 (CH_2C=O(1*S*)); 29.00 (CH_2(acetal)(1*S*)); 28.22 (CH_2(acetal)(1*R*)); 26.04 (C(5'')(1*R*+1*S*)); 25.56 (C(9'')(1*R*+1*S*)); 25.32 (C(12'')(1*R*+1*S*)); 25.11 (Me(acetal)(1*S*)); 23.61 (Me(acetal)(1*R*)); 17.38 (C(15'')(1*R*+1*S*)); 16.29 (C(14'')(1*R*+1*S*)); 15.62 (C(13'')(1*R*+1*S*)); 13.95 (Me(ester)(1*R*)); 13.96 (Me(ester)(1*S*)). ESI-MS: 598.38 $[M+H]^+$; calculated: 597.75. Anal calc. for$C_{32}H_{47}N_5O_6$ (597.75) * 0.5 DMF: C, 63.00; H, 8.02; N, 12.15. Found: C, 63.25; H, 7.84; N, 12.33.

NL_8.1.^{1}3.0*P*(4b):0.0485 g, 0.0747 mmol, 14 %; diasteroisomeric mixture. TLC (SiO_2*60*, CH_2Cl_2/MeOH 95:5; v/v):R_f, 0.54. UV (MeOH):λ_{max} 343.0 nm (ε = 8.100 $mol^{-1}cm^{-1}$). logP_{OW}(ALOGPS 3.01): 3.05 ± 0.74. ^{1}H-NMR (500.13 MHz, DMSO-d_6), 8.98 (*s*, H-C(10)); 8.40 (*s*, H-C(5)); 5.44-5.37 (*m*, 4H, H_2-C(1''), H_2-C(11)); 5.31 (*dd*, ^{3}J(H-C(2'),H-C(1')) = 4.5, ^{3}J(H-C(2'),H-C(3')) = 6.5, H-C(2')); 5.19 (*t*, ^{3}J(HO-C(5'),H_2-C(5')) = 6.0, HO-C(5')); 5.13 (*d*, ^{3}J(H-C(1'),H-C(2')) = 4.5, H-C(1')); 5.01-5.00 (*m*, 2H, H-C(10''), H-C(6'')); 4.87 (*dd*, ^{3}J(H-C(3'),H-C(2')) = 7.0, ^{3}J(H-C(3'),H-C(4')) = 3.0, H-C(3')); 4.10-4.05 (*m*, 3H, H-C(4'), CH_2(ester)(1*R*)); 4.02 (*q*, ^{3}J(CH_2(ester),Me(ester)) = 7.5; CH_2(ester)(1*S*)); 3.54-3.43(*m*, ^{2}J(H'-CH_2(5'),H''-CH_2(5')) = -12.0, ^{3}J(HO-(5'),CH_2-C(5')) = 5.0, HO-C(5')); ^{3}J(H-

C(4‘),H_2-C(5‘)) = 5.0, H_2-C(5‘)); 3.25 (*s*, Me(12)); 2.47 (*t*, ^3J(CH_2(C=O),CH_2(acetal)) = 7.5, CH_2(C=O)); 2.07-1.83 (*m*, 10H, CH_2(4‘‘), CH_2(5‘‘), CH_2(8‘‘), CH_2(9‘‘), CH_2(acetal)); 1.78 (*s*, 3H, Me(13‘‘)(1*R*)); 1.77 (*s*, 3H, Me(13‘‘)(1*S*)); 1.62 (*s*, 3H, Me(14‘‘)(1*S*)); 1.60 (*s*, 3H, Me(14‘‘)(1*R*)); 1.51 (*s*, 3H, Me(12‘‘)); 1.50 (*s*, 3H, Me(15‘‘)); 1.30 (*s*, 3H, Me(acetal)(1*R*)); 1.20 (*t*, ^3J(Me(ester),CH_2(ester)) = 7.5, Me(ester)(1*R*)); 1.14 (*t*, ^3J(Me(ester),CH_2(ester)) = 7.5, Me(ester)(1*S*)).^{13}C-NMR (125.76 MHz, DMSO-d_6), 172.53 (C=O(ester)); 157.18 (C(10)); 154.51 (C(9)); 151.06 (C(5)); 141.25 (C(3)); 140.86 (C(3a)); 138.31 (C(3‘‘)); 134.52 (C(7‘‘)); 130.46 (C(11‘‘)); 125.82 (C(2’’)); 123.98 (C(6‘‘)); 123.41 (C(10’’)); 120.43 (C(7a)); 114.05 (C(acetal)); 84.93 (C(1’)); 83.00 (C(4’)); 82.28 (C(2’)); 79.34 (C(3’)); 61.72 (C(5’)); 59.76 (CH_2(ester)); 48.88 (C(11)); 40.85 (C(12)); 39.04 (C(1’’)); 39.00 (C(4’’)); 38.64 (C(8’’)); 33.59 (CH_2C=O); 28.23 (CH_2(acetal)); 26.06 (C(5’’)); 25.57 (C(9’’)); 25.32 (C(12’’)); 23.65 (Me(acetal)); 17.37 (C(15’’)); 16.30 (C(14’’)); 15.64 (C(13’’)); 13.97 (Me(ester)). ESI-MS: 653.50 $[M+H+H_2]^+$;calculated:649.28. Anal. calc. for$C_{36}H_{51}N_5O_6$ (649.28) * 2 MeOH: C, 63.93; H, 8.33; N, 10.79. Found: C, 63.92; H, 8.06; N, 10.89.

3-{4-[7-Amino-1-(3-methyl-but-2-enyl)-1H-pyrazolo[4,3-d]pyrimidin-3-yl]-6-hydroxymethyl-2-methyl-tetrahydro-furo[3,4-d][1,3]dioxol-2-yl}-propionic acid ethyl ester (**3a**, **NL_8.1.^{1}1.0**) andEthyl-*3-{4-Hydroxymethyl-2-methyl-6-[8-methyl-1-(3-methyl-but-2-enyl)-1,6-dihydro-1,2,4,5a,9-pentaaza-cyclopenta[a]naphthalen-3-yl]-tetrahydro-furo[3,4-d][1,3] dioxol-2-yl}-propionate* (**4a**, **NL_8.1.^{1}1.0*P***). Anhydrous **NL_8.1.0.0** (**2**, 0.2 g; 0.534 mmol) was dissolved in dry and amine-free dimethylformamide (DMF, 6 ml). To this solution dry K_2CO_3 (0.24 g; 1.7 mmol) was added, and the suspension was stirred for 30 min at ambient temperature. Then, isopentenyl bromide (0.2 mL; 1.5 mmol) was added dropwise under N_2 atmosphere, and the resulting mixture was stirred for 24 h at room temperature. The salt was filtered off and washed three times with CH_2Cl_2. The filtrate and washings were combined and evaporated on a rotary evaporator. The resulting yellowish

oil was co-evaporated repeatedly with CH_2Cl_2 (water bath below 35 °C!) and then dried in high vacuo overnight.Separation and purification ofthe two title compounds was performed by silica gel flash chromatography (SiO_2*60*, column: 5.5 x 14 cm; CH_2Cl_2/MeOH 95:5; v/v; 0.3 bar). Two compounds with slightly different mobilities were isolated upon evaporation ofthe appropriate fractions. (i) Colorless foam (**3a**, **NL_8.1.^{1}1.0**) and aslightly yellowish oil, respectively(**4a**, **NL_8.1.^{1}1.0*P***).

NL_8.1.^{1}1.0(**3a**):0.10 g, 0.217 mmol, 41 %; diastereoisomeric mixture;TLC (SiO_2*60* (CH_2Cl_2/MeOH 95:5; v/v): R_f0.50. UV (MeOH):λ_{max} 300.4 nm (ε = 9.200 mol^{-1} cm^{-1}). logP_{OW}(ALOGPS 3.01): 1.02 ± 0.74. ^{1}H-NMR (500.13 MHz, DMSO-d_6), 8.17 (*s*, H-C(5)); 7.26 (*s*, br, NH_2); 5.28-5.25 (*m*, H-C(2'), HO-C(5'), H-C(2'')); 5.17 (*d*, ^3J(H_2-C(1''),H-C(2'')) = 6.5, H_2-C(1'')); 5.08 (*d*, ^3J(H-C(1'),H-C(2')) = 4.5, H-C(1')); 4.86 (*dd*, ^3J(H-C(3'),H-C(2')) = 7.0, ^3J(H-C(3'),H-C(4')) = 4.0, H-C(3')); 4.09-4.05 (*m*, 3H, CH_2(ester), H-C(4')); 3.54-3.43 (*m*, CH_2(5')); 2.46 (*t*, ^3J(CH_2(C=O),CH_2(acetal)) = 7.5, CH_2(C=O)); 2.05 (*t*, ^3J(CH_2(acetal),CH_2(C=O)) = 7.5, CH_2(acetal)); 1.77 (*s*, Me(4'')); 1.68 (*s*, Me(5'')); 1.29 (*s*, Me(acetal)); 1.20 (*t*, ^3J(Me(ester),CH_2(ester)) = 7.0, Me(ester)).^{13}C-NMR (125.76 MHz, DMSO-d_6), 172.48 (C=O(ester)); 151.46 (C(5)); 141.09 (C(3)); 140.92 (C(3a)); 136.01 (C(3'')); 121.29 (C(7a)); 119.87 (C(2'')); 114.02 (C(acetal)); 84.9 (C(1')); 82.94 (C(4')); 82.27 (C(2')); 79.84 (C(3')); 61.76 (C(5')); 59.77 (CH_2(ester)); 49.39 (C(1'')); 33.62 (CH_2(C=O)); 28.25 (CH_2(acetal)); 25.21 (Me(5'')); 23.65 (Me(acetal)); 17.95 (Me(4'')); 13.97 (Me(ester)). ESI-MS: 462.30 $[M+H+H_2]^+$; calculated: 461.51.

NL_8.1.^{1}1.0*P*(**4a**):0.055 g, 0.107 mmol, 20 %; diasteroisomeric mixture; TLC (SiO_2*60,*CH_2Cl_2/MeOH = 95:5; v/v): R_f 0.54. UV (MeOH):λ_{max} 343.0 nm (ε = 19.000 mol^{-1} cm^{-1}). logP_{OW}(ALOGPS 3.01): 1.24 ± 0.74. ^{1}H-NMR (500.13 MHz, DMSO-d_6), 8.98 (*s*, H-C(10)); 8.40 (*s*, H-C(5)); 5.44-5.381(*m*, 4H, H_2-C(1''), H_2-C(11)); 5.32 (*dd*, ^3J(H-C(2'),H-C(1')) = 4.5, ^3J(H-C(2'), H-C(3')) = 6.5, H-C(2')); 5.18 (*t*, ^3J(HO-C(5'),H_2-C(5')) = 7.0, HO-

C(5')); 5.12 (*d*, ^3J(H-C(1'),H-C(2')) = 5.0, H-C(1')); 4.87 (*dd*, ^3J(H-C(3'),H-C(2')) = 7.5, ^3J(H-C(3'),H-C(4')) = 3.0, H-C(3')); 4.10-4.04 (*m*, 3H, H-C(4'), CH_2(ester)(1*R*)); 4.02 (*q*, ^3J(CH_2(ester),Me(ester)) = 7.5, CH_2(ester)(1*S*)); 3.55-3.43 (*m*, H_2-C(5')); 3.26 (*s*, Me(12)); 2.47 (*t*, ^3J(CH_2(C=O),CH_2(acetal)) = 7.5, CH_2(C=O)); 2.06 (*t*, ^3J(CH_2(acetal),CH_2(C=O)) = 7.5, CH_2(acetal)); 1.79 (*s*, Me(4'')); 1.68 (*s*, Me(5'')); 1.30 (*s*, Me(acetal)); 1.20 (*t*, ^3J(Me(ester),CH_2(ester)) = 7.5, Me(ester)(1*R*)); 1.14 (*t*, ^3J(Me(ester),CH_2(ester)) = 7.5, Me(ester)(1*S*)).^{13}C-NMR (125.76 MHz, DMSO-d_6), 172.55 (C=O(ester)); 157.24 (C(10)); 154.54 (C(9)); 151.10 (C(5)); 141.24 (C(3)); 140.83 (C(3a)); 135.26 (C(3'')); 125.79 (C(2'')); 120.54 (C(7a)); 114.13 (C(acetal)); 84.92 (C(1')); 82.92 (C(4')); 82.23 (C(2')); 79.27 (C(3')); 61.70 (C(5')); 59.70 (CH_2(ester)); 52.02 (C(11)); 40.89 (C(12)); 34.93 (C(1'')); 33.58 (CH_2(C=O)); 28.24 (CH_2(acetal)); 25.17 (C(5'')); 23.68 (Me(acetal)); 17.97 (C(4'')); 13.98 (Me(ester)). ESI-MS: 517.38 $[M+H+H_2]^+$; calculated:513.59.

Ethyl 3-((2S,3aR,4R,6S,6aS)-4-(hydroxymethyl)-2-methyl-6-(7-oxo-6,7-dihydro-1H-pyrazolo[4,3-d] pyrimidin-3-yl)tetrahydrofuro[3,4-d][1,3]dioxol-2-yl) propanoate (**6**, **NL_9.1.0.0**, diastereoisomeric mixture). Anhydrous formycin B (**5**, **NS_9.0.0.0**; 0.50 g; 1.864 mmol) was dissolved in dry dimethylformamide 12 ml) and ethyl levulinate (0.50 ml; 3,542 mmol), triethyl orthoformate (0.46 ml; 2,8 mmol) and 4 M HCl in 1.4-dioxane (1.7 ml) were added. After stirring for 24h at ambient temperature, the resulting mixture was partitioned between CH_2Cl_2 (90 ml) and a saturated aqueous $NaHCO_3$ solution (20 ml). After separation ofthe phases the aqueous layer was extracted three times with CH_2Cl_2(30, 20, and 15 ml). The combined organic layers were evaporated on rotary evaporator followed by repeated co-evaporation with $CH_2Cl_2$to remove residual DMF. The resulting colorless oil was dried in high vacuo overnight at 40 °C. Purification ofthe title compound was performed by silica gel chromatography (SiO_2*60*, column: 6.5 x 11.5 cm; CH_2Cl_2/MeOH 9:1; v/v). Product-containing fractions were pooled and evaporated giving a

colorless foam (0.472 g, 1.2 mmol, 64 %). TLC (SiO_2*60* (CH_2Cl_2/MeOH 9:1; v/v): R_f 0.51. UV (MeOH):λ_{max} 278.5 nm (ε = 7.500 mol^{-1} cm^{-1}). logP_{OW}(ALOGPS 3.01): 0.05 ± 0.74. ^{1}H-NMR (500.13 MHz, DMSO-d_6): 14.18 (*s*, br., (H-N(6)); 12.30 (*s*, br., (H-N(1)); 7.88 (*s*, 1H, H-C(5)); 5.28 (*t*, 1H, ^3J(HO-C(5'),H_2-C(5')) = 5.0, HO-C(5')(1*R*)); 5.20 (*t*, 1H, ^3J(HO-C(5'),H_2-C(5')) = 5.0, HO-C(5')(1*S*)); 5.15-5.10 (*m*, 1H, H-C(1')); 4.92 (*s*, br., H-C(2')); 4.81 (*dd*, 1H, ^3J(H-C(3'),H-C(2')) = 6.5, ^3J(H-C(3'),H-C(4')) = 3.0, H-C(3')(1*R*)); 4.74 (*dd*, 1H, ^3J(H-C(3'),H-C(2')) = 6.5, ^3J(H-C(3'),H-C(4')) = 3.0, H-C(3')(1*S*)); 4.57 (*q*, 2H, ^3J(CH_2(ester),CH_3(ester)) = 7.5, CH_2(ester)(1*R*)); 4.01 (*q*, 2H, ^3J(CH_2(ester),CH_3(ester)) = 7.5, CH_2(ester)(1*S*)); 2.42 (*t*, 2H, ^3J(CH_2(C=O),CH_2(acetal)) = 7.5, CH_2(C=O)(1*R*)); 2.88 (*t*, 2H, ^3J(CH_2(C=O),CH_2(acetal)) = 7.5, CH_2(C=O)(1*S*)); 2.04 (*t*, 2H, ^3J(CH_2(acetal),CH_2(C=O)) = 7.5, CH_2(acetal)(1*R*)); 1.88 (*t*, 2H, ^3J(CH_2(acetal),CH_2(C=O)) = 7.5, CH_2(acetal)(1*S*)); 1.47 (*s*, 3H, CH_3(acetal)(1*S*)); 1.282 (*s*, 3H, CH_3(acetal)(1*R*)); 1.18 (*t*, 3H, ^3J(CH_3(ester),CH_2(ester)) = 7.5, CH_3(ester)(1*R*)); 1.12 (*t*, 3H, ^3J(CH_3(ester),CH_2(ester)) = 7.5, CH_3(ester)(1*S*)). ^{13}C-NMR (125.76 MHz, DMSO-d_6): 172.82 (C=O(ester)(1*R*)); 172.70 (C=O(ester)(1*S*)); 153.07 (C(7)(1*R*+1*S*)); 143.34 (C(5)(1*R*+1*S*)); ~143.3 (C(3)(1*R*+1*S*), superimposed by C(5)); 137.13 (C(3a)(1*R*+1*S*)); 127.81 (C(7a)(1*R*+1*S*)); 114.51 (C(acetal)(1*S*)); 144.31 (C(acetal)(1*R*)); 85.59 (C(4')(1*S*)); 85.19 (C(4')(1*R*)); 83.92 (C(1')(1*S*)); 83.16 (C(1')(1*R*)); 82.98 (C(3')(1*S*)); 82.29 (C(3')(1*R*)); 79.31 (C(2')(1*S*)); 78.74 (C(2')(1*R*)); 61.81 (C(5')(1*S*)); 61.69 (C(5')(1*R*)); 59.00 (CH_2(ester)(1*R*)); 59.97 (CH_2(ester)(1*S*)); 33.65 (CH_2C=O(1*R*+1*S*)); 29.19 (CH_2(acteal)(1*S*)); 28.35 (CH_2(acetal)(1*R*)); 25.36 (Me(acetal)(1*S*)); 23.83 (Me(acetal)(1*R*)); 14.13 (CH_3(ester)(1*R*)); 14.06 (CH_3(ester)(1*S*)).ESI-MS: 395.16 $[M+H]^+$, 789.25 $[2M+H]^+$; calculated: 394.38.

Ethyl 3-((2S,3aR,4R,6S,6aS)-4-(hydroxymethyl)-2-methyl-6-(7-oxo-1,6-bis((2E,6E)-3,7,11-trimethyl dodeca-2,6,10-trien-1-yl)-6,7-dihydro-1H-pyrazolo[4,3-d]pyrimidin-3-

yl)tetrahydrofuro[3,4-d][1,3]dioxol-2-yl) propanoate (**7**, **NL_9.1.^{1}3^{6}3.0**, diastereoisomeric mixture). To a solution of anhydrous **NL_9.1.0.0** (**6**, 0.2 g; 0.507 mmol) in dry and amine-free dimethylformamide (6 ml) dry K_2CO_3 (0.23 g; 1.6 mmol) was added, and the suspension was stirred for 30 min at room temperature. Subsequently, farnesyl bromide (0.2 ml; 1.5 mmol) was added dropwise under N_2 atmosphere. After stirring of the reaction mixture for 23h at ambient temperature the salt was filtered off and washed thrice with CH_2Cl_2.The combined organic phases were evaporated and the resulting yellowish oil co-evaporated repeatedly from CH_2Cl_2 to remove residual DMF and dried in high vacuo overnight. Purification ofthe title compound was achieved by silica gel chromatography (SiO_2*60*, column: 6.5 x 14 cm; CH_2Cl_2/MeOH 98:2; v/v). Upon evaporation of the main zone a colorless foam ofthe title compound was obtained (0.275 g, 0.342 mmol, 67.5 %). TLC (SiO_2*60* (CH_2Cl_2/MeOH 98:2; v/v)): R_f 0.44. UV (MeOH):λ_{max} 277.75 nm (ε = 7.200 mol^{-1} cm^{-1}). logP_{OW}(ALOGPS 3.01): 7.08 ± 0.74. In the following, H and C atoms of the farnesyl group at N(1) are indicated with "$_a$"; H and C atoms of the farnesyl moiety at N(6) are indicated "$_b$". ^{1}H-NMR (500.13 MHz, DMSO-d_6): 8.11 (*s*, H-C(5')(1*R*)); 8.11 (*s*, H-(5')(1*S*)); 5.31 (*t*, ^3J(HO-C(5'),H$_2$-C(5')) = 6.0, HO-C(5')(1*R*+1*S*)); 5.24-5.19 (*m*, H-C(2')(1*R*+1*S*)); 5.13 (*d*, ^3J(H-C(1''),H-C(2'')) = 7.0, H-C(1''$_a$)); 5.02 (*d*, ^3J(H-C(1'),H-C(2')) = 4.5, H-C(1')(1*R*+1*S*)); 4.98-4.93 (*m*, 6H, H-C(10''$_{a,b}$), H-C(6''$_{a,b}$), H-C(2''$_{a,b}$)); 4.79 (*dd*, ^3J(H-C(3'),H-C(2')) = 7.0, H-C(3')(1*R*)), ^3J(H-C(3'),H-C(4')) = 3.5, H-C(3')(1*R*)); 4.57 (*d*, ^3J(H-C(1''),H-C(2'')) = 7.0, H-C(1''$_b$)); 4.07-4.00 (*m*, 3H, H-C(4'), CH$_2$(ester)); 3.46-3.40 (*m*, CH$_2$(5')(1*R*+1*S*)); 2.43 (*t*, ^3J(CH$_2$C=O,CH$_2$(acetal)) = 7.0, CH$_2$C=O(1*R*)); 2.27 (*t*, ^3J(CH$_2$C=O,CH$_2$(acetal)) = 7.0, CH$_2$C=O(1*S*)); 2.04-1.80 (*m*, 17H, CH$_2$(4''$_{a,b}$), CH$_2$(5''$_{a,b}$), CH$_2$(8''$_{a,b}$), CH$_2$(9''$_{a,b}$), CH$_2$(acetal)); 1.76 (*s*, Me(13''$_{a,b}$)); 1.58-1.57 (*m*, 6H, Me(14''$_{a,b}$)); 1.49-1.47 (*m*, 12H, Me(12''$_{a,b}$), Me(15''$_{a,b}$)), 1.29 (*s*, Me(acetal)(1*S*)); 1.27 (*s*, Me(acetal)(1*R*)); 1.18 (*t*, ^3J(Me(ester),CH$_2$(ester)) = 7.0, Me(ester)(1*R*)); 1.12 (*t*,

^{3}J(Me(ester),CH_2(ester)) = 7.0, Me(ester)(1*R*)).^{13}C-NMR (125.76 MHz, DMSO-d_6): 172.82 C=O(ester)(1*R*); 172.71 (C=O(ester)(1*S*)); 152.64 (C(7)(1*R*+1*S*)); 146.09 (C(3)(1*R*+1*S*)); 141.96 (C(5)(1*R*+1*S*)); 140.57 (C(3''$_a$)); 139.75 (C(3''$_b$)); 136.80 (C(3a)(1*R*+1*S*)); 134.83, 134.78 (C(7''$_{a,b}$)); 134.72 (C(7a)(1*R*+1*S*)); 130.72, 130.711 (C(11''$_{a,b}$)); 124.17, 124.10 (C(6''$_{a,b}$)); 123.54, 123.53 (C(10''$_{a,b}$)); 119.46, 119.10 (C(2''$_{a,b}$)); 114.41 (C(acetal)(1*R*)); 114.21 (C(acetal)(1*S*)); 85.4 (C(1')(1*S*)); 85.17 (C(1')(1*R*)); 83.19 (C(4')(1*R*)); 83.08 (C(4')(1*S*)); 82.39 (C(2')(1*R*)); 81.50 (C(2')(1*S*)); 79.31 (C(3')(1*S*)); 78.77 (C(3')(1*R*)); 61.87 (C(5')(1*S*)); 61.72 (C(5')(1*R*)); 60.05 (CH_2(ester)(1*R*)); 60.01 (CH_2(ester)(1*R*)); (C(1''$_{a,b}$)(1*R*+1*S*)), (C(4''$_{a,b}$)(1*R*+1*S*)) und (C(8''$_{a,b}$)(1*R*+1*S*)), superimposed by (D_6)DMSO; 33.64 (CH_2(C=O)(1*R*+1*S*)); 29.20 (CH_2(acetal)(1*S*)); 28.37 (CH_2(acetal)(1*R*)); 26.25, 26.20 (C(5''$_{a,b}$)); 25.99, 25.64 (C(9''$_{a,b}$)); 25.50, 25.38 (C(12''$_{a,b}$)); 23.82 (Me(acetal)(1*R*)); 22.13 (Me(acetal)(1*S*)); 17.54, 17.51 (C(15''$_{a,b}$)); 16.37, 16.30 (C(14''$_{a,b}$)); 15.87, 15.85 (C(13''$_{a,b}$)); 14.14 (Me(ester)(1*R*)); 14.09 (Me(ester)(1*S*)).ESI-MS: 804.08 $[M+H]^+$; calculated: 803.10.

5.4. Cells and culture conditions

Cell viability analyses wereperformed with rat malignant neuroectodermal BT4Ca cells [13, 15] (a kind gift from Dr. *N. John*, Hannover Medical School, Hannover, Germany), human glioblastoma GOS-3 cells (DSMZ, GmbH, DE-Braunschweig), human glioblastoma cells U-87MG (ATCC® HTB14™ purchased from LGC Standards GmbH, Wesel, Germany),patient-derived human primary glioblastoma cells GBM 2014/42, and with human acute monocytic leukemia cell line THP-1 (DSMZ, GmbH, Braunschweig,Germany). As described earlier [15, 16, 17, 20], theTHP-1 cells were cultured in 90% RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 0.1 mg/ml streptomycin and were grown at 37°C in a humidified atmosphere (5% CO2, 95% air).U87MG cells were cultivated in DMEM with 10% FCS. Patient derivedprimary

glioblastoma cells GBM 2014/42 wereobtained from a 53-year old male patient. In routine pathological analysis no methylation of the MGMT promotor was found. Tumor tissue was washed in HEPES-buffered saline, homogenized and treated for 30 min with 0.025% Trypsin/EDTA solution at 37° C. The resulting cell homogenate was passed over an 80mm cell strainer and the cell suspension was centrifuged (200g, 5 min.). After 2 washes with medium (DMEM, 10% FCS), the cells were seeded out for propagation and kept under differentiating conditions.Collection and processing of primary human GBM WHO° IV tumor samples were in accordance with the ethical standards of the Helsinki Declaration in 2008. Informed consent of patients was obtained for acquisition, processing, and documentation of pseudonymized samples as approved by the local ethics committee (Ref No: 185/11, Medical Faculty, Marburg University).

5.5. Measurements of survival/viability of 5-FUrd and Formycin A/Bderivatives

Cells were seeded onto 96-well plates (BD FalconTM, *Becton Dickinson* GmbH, Heidelberg, Germany) at densities of 4x 10^4(THP1), 1.5 x 10^4(GOS-3) or 5 x 10^3(BT4Ca, U-87 MG, GBM 2014/42) cells per well. 5-FUrd (NS_4.0.0.0), Formycin A (**NS_8.0.0.0**) and its derivatives **NL_8.1.0.0, NL_8.1.^{1}3.0, NL_8.1.^{1}3.0 P** and **NL_8.1.^{1}1.0 P** as well as Formycin B (**NS_9.0.0.0**) and its derivatives **NL_9.1.0.0** and **NL_9.1.^{1}3^{6}3.0**were tested at concentrations of 1.56, 3.12, 6.25, 12.5, 25 or 50µM. The survival/viability was measured after 48h using PrestoBlue® reagent (*Invitrogen-Life Technologies* GmbH, *Darmstadt*, Germany) as described earlier [15, 16, 17,18, 19, 20]. The reagent was given into the culture medium at a final concentration of 10%. After 30min, 1h, 2h or 3h the optical densities (OD) were measured at 570 and 600nm (reference) with a SUNRISE ELISA-reader (*Tecan*, *Salzburg*, Austria). Results were calculated in % of the viability (OD570/600nm of the samples x 100 / OD570/600nm of the control without substances). As negative control (=100% viability =0% cytotoxicity), cells were cultured with medium alone.

To exclude a possible cytotoxic effect of DMSO, which was used as solvent for 5-FUrd and all other test compounds, cells were incubated in medium plus DMSO (=0.05% DMSO = equal to DMSO concentration for a 50µM substance dilution) to produce a ”DMSO” control. To measure possible cytotoxic (side)effects- of the substances under test - on immune cells, weperformedexperiments with PMA(100 ng/ml; 72h) differentiated human THP-1 macrophages. After PMA-mediated differentiation, the human THP-1 macrophages were incubated (48 h) with six different compound concentrations to assess cell viability. Alongside all viability assays, cells of different origin were incubated with DMSO or 5-FUrd to generatea “DMSO”- and a “positive” control.

Acknowledgements

The authors gratefully acknowledge NMR measurements by Mrs. *M. GatherSteckhan*, ESI MS measurements by Dr. *S. Walter*, and elemental analyses by Mrs. *A. Schuster*.Moreover, we thank Prof. Dr. *U. Beginn* for excellent laboratory facilities. We additionally thank *A. Cordes* and *N. Heinrich* for excellent technical assistance.

Author Contribution Statement:

C. Knies and H. Rosemeyer performed the syntheses; E. Bender conducted the biophysical experiments;K. Hammerbacher carried out the cell-biological experiments;R. Hannen and J. W. Bartsch isolated, cultured and established the primary glioblastoma cells (GBM 2014/42) and provided the U-87 MG cell line; K. Hammerbacherand G. A. Bonaterra analyzed the cell-biological results; H. Rosemeyer analyzed NMR-spectroscopic and biophysical data; H. Rosemeyer and R. Kinscherf conceived and designed the experiments; H. Rosemeyer, J. W. Bartsch, C. Nimsky and R. Kinscherf wrote the paper.

References and Notes

[1] A. Bzwoska ,`Formycins and their Analogues: Purine Nucleoside Phosphorylase Inhibitors and their Potential Applicationin Immunosuppression and Cancer´, in: Modified Nucleosides in Biochemistry, Biotechnology and Medicine, P. Herdewijn (Ed.), Wiley-VCH, Weinheim, 2008, pp. 473-504.

[2] C. Knies, PhD Thesis, University of Osnabrück, **2017**, pp. 53 ff.C. Knies, PhD thesis, University of Osnabrück, 2017, Repository of the university library: **urn:nbn:de:gbv:700-2017042115834**.

[3] W. J. Malaisse, P. Lebrun, B. Pirotte, P. D. Van Poelje, C. Viйambres, M. L. Villanueva-Peйacarillo, I. Valverde, J. Gäbel, P. Rorsman, `The Riddle of Formycin A Insulinotropic Action´, *Biochemical and Molecular Medicine***1996**, *57*, 47-63.

[4] J. Giziewicz, E. deClercq, M. Luczak, D. Shugar, `Antiviral and Antimetabolic Activities of Formycin and its N_1, N_2, 2'-O- and 3'-O-Methylated Derivatives´, *Biochem. Pharmacol*. **1975**, *24*, 1813-1817.

[5] F. Seela, Y. Chen, A. Melenewski, H. Rosemeyer, C. Wei, `Synthesis and application of novel nucleoside phosphonates and phosphoramidites modified at the base moiety´, *Acta Biochim. Pol*. **1996**, *43*, 45-52.

[6] K. Mühlegger, H. von der Eltz, F. Seela, H. Rosemeyer, `C-Nucleoside Derivatives and their Use in Nucleic Acid Detection´, WO 96/28460, September 19, 1996.

[7] D. E. Clark, ´Rapid calculation of polar molecular surface area and its application to the prediction of transport phenomena. 1. Prediction of intestinal absorption. ´*J. Pharm. Sci*. **1999**, *88*, 807-814.

[8] M. D. Wessel, P. C. Jurs, J. W. Tolan, S. M. Muskal, ´Prediction of human intestinal absorption of drug compounds from molecular structure`,*J. Chem. Inform. Comp. Sci.***1998**, *38*, 726-735.

[9] I. V. Tetko, G. I. Poda, C. Ostermann, R. Mannhold, `Large Scale Evaluation of log *P* Predictors: Local Corrections May Compensate Insufficient Accuracy and Need of Experimentally Testing Every Other Compound´,*Chem. Biodiversity***2009**, *6*, 1837-1844.

[10] R. Mannhold, G. I. Poda, C. Ostermann, I. V. Tetko, `Calculation of Molecular Lipophilicity: State-of-the-Art and Comparison of log *P* Methods on more than 96,000 Compounds´,*J. Pharmaceutical Sci.***2009**, *98*, 861-893.

[11] P. Seiler, `Interconversion of Lipophilicities from Hydrocarbon/Water Systems into the Octanol/Water System´, *Eur. J. Med. Chem*. **1974**, *9*, 473-479.

[12] R. C. Young, R. C. Mitchell, T. H. Brown, C. R. Ganellin, R. Griffiths, M. Jones, K. K. Rana, D. Sounders, I. R. Smith, N. E. Sore, T. J. Wilks, `Development of a New Physicochemical Model for Brain Penetration and its Application to the Design of Centrally Acting H2 Receptor Histamine Antagonists´, *J. Med. Chem*. **1988**, *31*, 656-671.

[13] O. D. Laerum, M. F. Rajewsky,M.Schachner,D.Stavrou,K. G. Hablid,A.Haugen, `Phenotypic Properties of Neoplastic Cell Lines Developed from Fetal Rat BrainCells in Culture After Exposure to Ethylnitrosourea in vivo´, *Z. Krebsforsch.,Klin. Onkol. Cancer Res. Clin. Oncol.***1977**, *89*,273-295.

[14] C. Knies, H. Rosemeyer, https://www.researchgate.net/profile/Helmut_Rosemeyer and urn:nbn:de:gbv:700-2015110413639. The publication can be downloaded free

from: https://repositorium.uni-osnabrueck.de/handle/urn:nbn:de:gbv:700-2015110413639.

[15] C. Knies, K. Hammerbacher, G. A. Bonaterra, R. Kinscherf, A. Rosemeyer, `Novel Nucleolipids of Pyrimidine ß-D-Ribonucleosides: Combinatorial Synthesis, Spectroscopic Characterization, and Cytostatic/Cytotoxic Activities´, *Chem. Biodiversity***2016**, *13*, 160-180.

[16] C. Knies, G. A. Bonaterra, K. Hammerbacher, A. Cordes, R. Kinscherf, H. Rosemeyer, `Ameliorated or Acquired Cytostatic/Cytotoxic Properties of Nucleosides by Lipophilization´, *Chem. Biodiversity***2015**, *12*, 1902-1944.

[17] C. Knies, K. Hammerbacher, G. A. Bonaterra, R. Kinscherf, H. Rosemeyer, `Nucleolipids of Canonical Purine ß-D-Ribonucleosides: Synthesis and Cytostatic/CytotoxicActivities Toward Human and Rat GlioblastomaCells´,*ChemistryOpen***2015**, *5*, 129-141.

[18] A. Farhat, E. Malecki, G. A. Bonaterra, D. Röthlein, M. Wolf, J. Schmitt, H. Rosemeyer, R. Kinscherf, `Cytostatic/Cytotoxic Effects of 5-Fluorouridine Nucleolipids on Colon, Hepatocellular, and Renal Carcinoma: in vitro Identification of a Potential Cytotoxic Multi-Anticancer Drug´,*Chem. Biodiversity***2014**, *11*, 469-482.

[19] E. Malecki, A. Farhat, G. A. Bonaterra, D. Röthlein, M. Wolf, J. Schmitt, R. Kinscherf, H. Rosemeyer, `Synthesis of 5-Fluorouridine Nucleolipid Derivatives and Their Cytostatic/Cytotoxic Activities on Human HT-29 Carcinoma Cells´, *Chem. Biodiversity* **2013**, *10*, 2235-2246.

[20] K. Hammerbacher, K. Görtemaker, C. Knies, E. Bender, G.A. Bonaterra, H. Rosemeyer, R. Kinscherf, `Combinatorial Synthesis of Novel Pyrimidine- and Purine-ß-*D*-Ribonucleoside Nucleolipids: Their Distribution Between Aqueous and Organic

Phases and Their *in vitro* Activity Against Human- and Rat Glioblastoma Cells *in vitro´*,*Chem. Biodiversity*.**2018** Sep;15(9):e1800173.

10.1002/cbdv.201900012

Table of Contents

Nucleolipids of the Nucleoside Antibiotics Formycin A and B: Synthesis and Biomedical Characterization

Helmut Rosemeyer [1]) Christine Knies [1]), Eugenia Bender [1]), Katharina Hammerbacher [2]), Gabriel A. Bonaterra[2]), Ricarda Hannen [3]), Jörg W. Bartsch [3]), Christopher Nimsky [3]), Ralf Kinscherf [2])

Nocardia interforma

http://www.nih.go.jp/saj/DigitalAtlas