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Neomycin–phenolic conjugates: Polycationic amphiphiles with broad-spectrum antibacterial activity, low hemolytic activity and weak serum protein binding

Brandon Findlay [a], George G. Zhanel [b], Frank Schweizer [a,b,*]

[a] Department of Chemistry, University of Manitoba, Winnipeg, MB, Canada R3T 2N2
[b] Department of Medical Microbiology, University of Manitoba, Health Sciences Centre MS673-820 Sherbrook St., Winnipeg, MB, Canada R3A 1R9

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ABSTRACT

Here we present a proof-of-concept study, combining two known antimicrobial agents into a hybrid structure in order to develop an emergent cationic detergent-like interaction with the bacterial membrane. Six amphiphilic conjugates were prepared by copper (I)-catalyzed 1,3-dipolar cycloaddition between a neomycin B-derived azide and three alkyne-modified phenolic disinfectants. Three conjugates displayed good activity against a variety of clinically relevant Gram positive and Gram negative bacteria, including MRSA, without the high level of hemolysis or strong binding to serum proteins commonly observed with other cationic antimicrobial peptides and detergents.

Over the years, many first-line antibiotics have been relegated to the back benches as an increased presence of drug resistant bacteria rendered them ineffective. At the same time, a reduced focus on antibiotic research at the major pharmaceutical companies has drastically reduced the rate of drug discovery, leaving us more in need of new antimicrobial agents and scaffolds than ever.[1,2] The need for new scaffolds is especially great, as widespread resistance to most antibiotics appears shortly after the drugs are introduced into clinical use, with a gap of roughly twenty years for penicillins and less than nine years for fluoroquinolones (five years for ciprofloxacin).[3,4] This resistance may affect many drugs with similar scaffolds, limiting the effectiveness of new drugs before they even enter the clinic. Resistance seems to arise from small numbers of bacteria already present in the population at large. Attempts to determine the age of common resistance mechanisms has found that, like the secondary metabolites many current antibiotics are based upon, the genes which code for antibiotic resistance are ancient.[5] The potential for widespread drug resistance is thus latent in every bacterial population, and limiting its emergence will require improved education in the use of antibiotics and the creation of antibiotic classes that have been designed with antimicrobial resistance in mind.

Most commercial antibiotics are molecular inhibitors, binding to enzymes, cellular receptors or nucleic acids within the cell and inhibiting their function. Bacterial resistance arises from mechanisms which disrupt the drug–target complex, either through modification of the binding site (via DNA mutation or chemical alteration), the drug (via acetylases, phosphates and others) or by simply preventing the drug from entering the cell and encountering its target (efflux pumps).[5,6] Drug efflux is of particular concern,

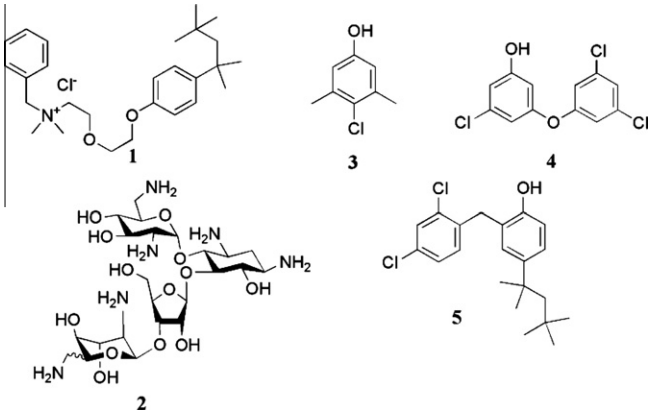

Figure 1. Benzethonium chloride, **1**, served as the initial template for the hybrids presented in this Letter. Neomycin B, **2**, was used for its cationic charges, RNA-binding properties and self-promoted uptake, whereas the phenolics chloroxylenol, **3**, triclosan, **4**, and clofoctol, **5**, are expected to induce hydrophobic membrane interactions in the hybrids.

* Corresponding author.
E-mail address: schweize@ms.umanitoba.ca (F. Schweizer).

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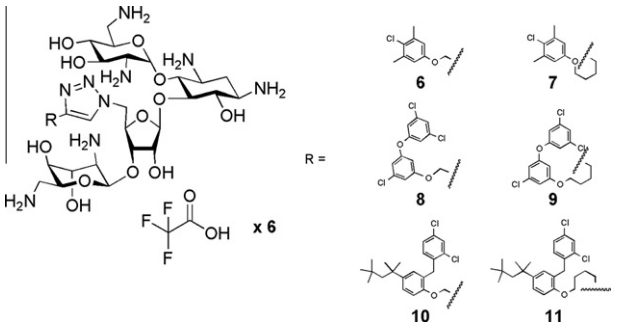
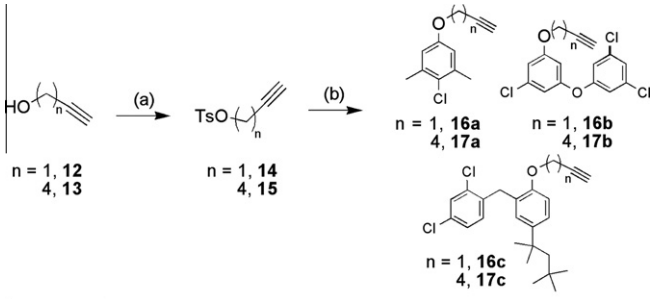


Figure 2. Structures of the neomycin–phenol conjugates produced in this study. Conjugates **6** and **7** are based upon chloroxylenol, conjugates **8** and **9** on triclosan and **10** and **11** on clofoctol. All six conjugates use neomycin B to provide the polycationic charge.

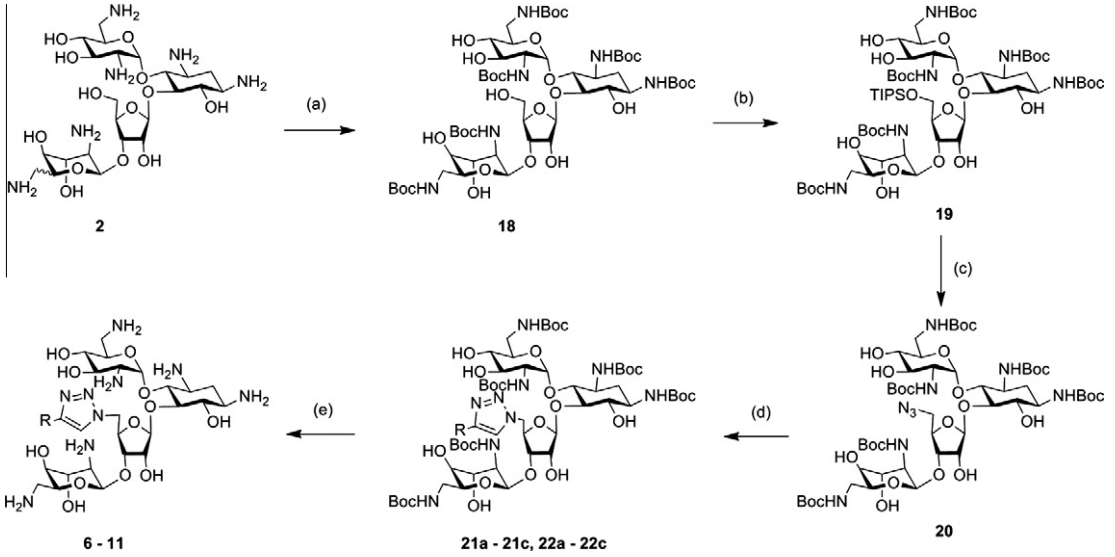


Scheme 1. Synthesis of the alkyne linkers. Conditions: (a) TsCl, KOH, Et_2O (41–64%). (b) **3**–**5**, K_2CO_3, DMF (51–96%).

as the poor selectivity of efflux pumps can easily lead to broad antibiotic resistance, with a single pump effective against whole classes of antibiotics.[7] Bacteria which endogenously express a large number of drug efflux pumps, such as *Pseudomonas aeruginosa*, are able to withstand most common antimicrobial agents and are often the cause of multi-drug resistant (MDR) infections.

As a means of circumventing these resistance mechanisms, work in our lab and others has explored the potential for (poly)cationic amphiphiles (CAs) such as antimicrobial peptides, lipids and surfactants to act as antimicrobial agents.[8–16] Found throughout nature and long used as antimicrobial detergents, cationic amphiphiles do not act on any single target within the cell, instead they disrupt DNA replication, protein synthesis and bacterial membrane integrity.[12] Widely varying in structure and size, all CAs are based on two common features: a hydrophobic face that interacts with the lipid bilayer and a polar, cationic face that is drawn via electrostatic interactions to anionic moieties such as some lipid head groups and nucleic acids. Little in vitro resistance to these amphiphiles has been observed, due to their multiple modes of action and ability to form pores in the bacterial membrane, but their clinical use has been severely limited due to issues with selectivity, protease susceptibility and toxicity.[17] The therapeutic ratio of these amphiphiles has been improved, reducing their ability to lyse red blood cells and increasing selectivity towards Gram positive and Gram negative bacteria.[18] Nevertheless, the presence of non-specific binding to human serum proteins remains a major limitation of these agents, resulting in loss of antibacterial activity in vivo.[19] Starting from the structure of an amphiphilic disinfectant, the quaternary ammonium compound benzethonium chloride, **1** (Fig. 1), we devised alternatives to the classical CAs, in order to create agents with similar characteristics but devoid of their limitations.[20] This work has cumulated in neomycin–phenolic conjugates presented here (Fig. 2).

Our conjugates use two known antimicrobial agents to create the cationic and hydrophobic faces required for interaction with bacterial membranes. The hybrid molecules are intended to display three distinct modes of action; one from each participating agent and one from an emergent CA-like behavior from the superstructure itself. This triple mode-of-action should lead to broad spectrum activity and resilience against bacterial resistance, as resistance to one agent will not alter susceptibility to the other half of the conjugate or to the CA-like mode of action. In optimal cases this will allow the conjugates to retain activity against even MDR bacteria. This strategy has been attempted previously, but has been hampered by the large size of the conjugates, which reduces diffusion across the cellular membrane.[21,22] To maintain permeability we chose phenolic disinfectants as the hydrophobic segment, as phenolics are known to derive at least part of their activity from moving small cations across the bacterial membrane, which requires rapid diffusion in and out of the cell.[20] When combined with the polycationic aminoglycoside neomycin, which is known



Scheme 2. Synthesis of conjugates **6**–**11**. Conditions: (a) Boc_2O (10 equiv), TEA/MeOH/H_2O (61%). (b) TIPS-Cl (31 equiv), pyridine (41%). (c) NaN_3 (10 equiv), DMF/H_2O (94%). (d) **16a**–**c**, **17a**–**c** (1.2 equiv), CuI (0.2 equiv), DIPEA (3 equiv), ACN (50–88%). (e) TFA/H_2O (80–91%).

Table 1
Antimicrobial activity[a] and hemolysis of the conjugates and drug standards

Organism	Compound										
	1	**2**[r]	**3**	**4**	**5**[s]	**6**	**7**	**8**	**9**	**10**	**11**
S. aureus[b]	2	1	32	0.5	—	16	16	32	4	8	16
MRSA[c]	2	256	64	⩽0.25	—	256	128	128	8	8	16
MSSE[d]	2	0.5	32	1	—	8	8	16	4	4	8
MRSE[e]	8	⩽0.25	16	⩽0.25	—	8	8	16	2	1	8
E. faecalis[f]	4	16	256	8	—	64	128	64	16	8	64
E. faecium[g]	4	4	256	16	—	128	16	16	8	8	8
S. pneumoniae[h]	2	32	128	128	—	64	64	256	64	64	64
E .coli[i]	32	4	256	⩽0.25	—	16	32	64	16	16	64
E. coli[j]	32	1	256	1	—	16	16	64	16	64	64
E. coli[k]	32	8	256	1	—	128	128	128	64	64	128
P. aeruginosa[l]	64	512	512	>512	—	512	512	512	128	128	128
P. aeruginosa[m]	64	256	256	64	—	256	256	256	64	64	64
S. maltophilia[n]	32	>512	128	512	—	>512	>512	>512	>512	512	512
A. baumannii[o]	32	64	128	8	—	>512	512	>512	128	64	256
K. pneumoniae[p]	32	0.25	256	1	—	8	4	32	4	64	32
Hemolysis[q]	77.3	0.69	—	—		0.75	0.75	0.99	1.62	27.5	22.5

[a] MIC_{90}, reported in μg/mL.
[b] ATCC 29213.
[c] ATCC 33592.
[d] 81388 CANWARD 2008.
[e] CAN-ICU 61589.
[f] ATCC 29212.
[g] ATCC 27270.
[h] ATCC 49619.
[i] ATCC 25922.
[j] CAN-ICU 61714.
[k] CAN-ICU 63074.
[l] ATCC 27853.
[m] CAN-ICU 62308.
[n] CAN-ICU 62584.
[o] CAN-ICU 63169.
[p] ATCC 13883.
[q] Percent hemolysis at 100 μg/mL of compound.
[r] Neomycin trisulfate hydrate.
[s] Compound **5** was poorly soluble in water and its activity could not be accurately assessed.

to self-promote its own uptake into bacterial cells by disrupting polysaccharide–cation interactions,[23] we expected our conjugates to display good diffusion kinetics, despite their larger size and high number of hydrogen bond donors and acceptors. Moreover, the RNA-binding motif of neomycin may induce intracellular modes of antibacterial action in the conjugates.[24]

Work with other CAs has found that altering the size and shape of the hydrophobic domain can greatly influence antimicrobial activity,[12,25] and so we linked three distinct phenolic disinfectants, **3–5**, to neomycin B to create the hybrid compounds **6**–**11** (Fig. 2). While triclosan, **4**, has been found to inhibit fatty acid synthesis by blocking the key enzyme FabI,[26] the targets of chloroxylenol, **3**, and clofoctol, **5**, are unknown. It appears that as a class the phenols, like CAs, have a number of cellular interactions but derive much of their activity from interactions with the bacterial membrane.[20] Easily ionized, the phenols seem to ferry small cations across the bacterial membrane, dispersing the membrane polarization. While we expect much of this activity to be inhibited by the ether linkage used to connect these phenols to neomycin B, an analysis of the structure of **1** suggests that interactions with the cellular membrane will be maintained, allowing the hybrids to pass through the membrane and maintain a high intracellular concentration.

To produce the conjugates the two antimicrobials were linked via a copper (I)-catalyzed 1,3-dipolar cycloaddition reaction between the phenol-modified alkynes (Scheme 1),[27,28] and the neomycin-based azide **20** (Scheme 2). The length of the phenolic alkyne linker was varied, and attached to the phenols by displacement of the corresponding alkyne sulfonate esters.[29] Azide-functionalized neomycin B was prepared as a Boc-protected derivative using previously established methodology.[10,14,30]

The single primary hydroxyl group of neomycin was selected as the point of attachment as previous studies have shown that modifications at this position allow the aminoglycoside to retain antibacterial activity and RNA-binding.[14,31]

Briefly, neomycin sulfate was treated with di-tert-butyl-dicarbonate in a mixture of methanol, water and triethylamine to protect the amino groups. Flash chromatography of crude **18** was then used to separate Boc protected neomycin B from neomycin C, and the primary hydroxyl moiety was activated using a large excess of triisopropylsulfonyl chloride (TIPS-Cl) to afford sulfonate ester **19**. Addition of sodium azide in a mixture of DMF and water at 70 °C cleanly produced the required azide **20**. The two halves of the conjugate were then linked through copper (I) catalyzed 1,3-dipolar cycloaddition. Deblocking with trifluoroacetic acid gave the neomycin–phenol conjugates **6**–**11**.

Antibacterial activity was assessed using macrobroth dilution assays according to standard CLSI methodology.[32] Compound activity was determined against a combination of reference strains of Gram-positive and Gram-negative bacteria and clinically relevant pathogens from the national surveillance CAN-ICU and CANWARD studies.[32,33] The inclusion of clinically relevant bacteria is especially important in light of the rapid increase in antimicrobial resistance with varied resistance mechanisms. Bacteria from current hospital environments are far more likely to be resistant to a variety of antibiotics and disinfectants with different chemical structures and mechanism(s) of action, and testing with only laboratory strains can produce misleadingly effective antimicrobial activities. Full results are summarized in Tables 1 and 2.

The most active hybrids were compounds **9** and **10**, one of which had triclosan as the phenolic with the longer of our two

Table 2
Antimicrobial activity[a] of conjugates and drug standards in the presence of bovine serum albumin (BSA)

Organism	Compound										
	1	**2**[q]	**3**	**4**	**5**[r]	**6**	**7**	**8**	**9**	**10**	**11**
S. aureus[b]	16	0.5	512	0.5	—	32	32	64	16	8	16
MRSA[c]	32	128	512	1	—	512	256	256	32	16	32
MSSE[d]	128	0.5	512	1	—	8	8	16	4	2	4
MRSE[e]	128	⩽0.25	512	1	—	8	8	16	2	4	4
E. faecalis[f]	32	16	>512	512	—	128	128	128	32	16	64
E. faecium[g]	32	4	>512	512	—	64	32	64	32	8	16
S. pneumoniae[h]	16	8	>512	256	—	128	256	512	128	128	64
E. coli[i]	256	0.25	>512	16	—	32	32	64	16	64	64
E. coli[j]	256	1	>512	16	—	16	16	64	8	128	128
E. coli[k]	256	16	>512	64	—	128	128	256	128	256	256
P. aeruginosa[l]	512	256	>512	>512	—	512	>512	512	512	512	512
P. aeruginosa[m]	512	256	>512	>512	—	256	256	256	256	128	256
S. maltophilia[n]	256	>512	>512	>512	—	>512	>512	>512	>512	>512	>512
A. baumannii[o]	256	32	>512	128	—	>512	512	>512	256	256	>512
K. pneumoniae[p]	256	⩽0.25	>512	32	—	4	4	32	2	64	32

[a] MIC_{90}, reported in μg/mL.
[b] ATCC 29213.
[c] ATCC 33592.
[d] 81388 CANWARD 2008.
[e] CAN-ICU 61589.
[f] ATCC 29212.
[g] ATCC 27270.
[h] ATCC 49619.
[i] ATCC 25922.
[j] CAN-ICU 61714.
[k] CAN-ICU 63074.
[l] ATCC 27853.
[m] CAN-ICU 62308.
[n] CAN-ICU 62584.
[o] CAN-ICU 63169.
[p] ATCC 13883.
[q] Neomycin trisulfate hydrate.
[r] Compound **5** was poorly soluble in water and its activity could not be accurately assessed.

linkers, and the other which had clofoctol and a short linker. Both molecules displayed similar or improved activity against the Gram positive bacteria in our study, with an improved activity of 64 μg/mL observed against the normally highly neomycin B resistant *P. aeruginosa* strain CAN-ICU 62308. Unlike many other CAs, compound **9** was not appreciably hemolytic at near MIC concentrations. The optimal spacing between cationic and hydrophobic domains appears to differ between hydrophobic domains and must be determined on a case-by-case basis.

Inspecting the antimicrobial results as a whole revealed a number of trends. The chloroxylenol conjugates **6** and **7** were broadly ineffective, displaying reduced activities compared to conventional neomycin sulfate. The triclosan conjugate with a short linker, **8**, was similarly inactive, though the longer triclosan conjugate **9** was more active than neomycin sulfate against MRSA and two *P. aeruginosa* strains. Both conjugates of clofoctol were active, but both displayed increased hemolytic activity, suggesting that the hydrophobic tail of clofoctol mediates non-specific interactions, similar to our standard CA, **1**. The intermediate hemolytic activity of **10** and **11** is likely due to the influence of the cationic aminoglycoside face, which may preferentially target the hybrids to negatively charged bacterial membrane lipids, reducing interactions with zwitterionic eukaryotic cells. In general, it appears that the conjugates are less active against neomycin susceptible bacteria such as MRSE and *Klebsiella pneumoniae*, but more active than neomycin against drug resistant strains such as MRSA and *P. aeruginosa*.

To test the influence of non-specific interactions on conjugate activity antimicrobial testing was repeated in the presence of 4% bovine serum albumin (BSA). Many cationic amphiphiles show greatly reduced activity in the presence of BSA due to protein binding,[19] but with the exception of the results for compounds **9**–**11** against *P. aeruginosa*, we observed little difference in activity. Our cationic amphiphile standard, **1**, in contrast had an average eight-fold reduction in efficacy, with the median Gram positive MIC rising from 2 to 16 μg/mL and the median Gram negative MIC moving from 32 to 256 μg/mL. This reduction was expected, given the importance of hydrophobicity on nonspecific membrane interactions. As they are not greatly inhibited by BSA, we infer that much of the antimicrobial effect in the conjugates is from the action of either neomycin B or the various phenolics. Interestingly, the shorter neomycin–triclosan conjugate **9** retains potent activity in the presence of BSA against two *Escherichia coli* strains (MIC ⩽ 16) while benzethonium chloride is only weakly active (MIC = 256) under these conditions.

Further characterization of the conjugates' mode of action can be determined by examining the activities of triclosan. Triclosan is known to possess several modes of action, with much of its activity stemming from inhibition of FabI, a key component of fatty acid synthesis.[26] Crystal structures of triclosan bound to FabI suggest that hydrogen bonding between the phenol and enzyme is important to enzyme binding.[34] While our unaltered triclosan standard had an MIC against *E. coli* ATCC 25922 of ⩽0.25 μg/mL, both triclosan conjugates **8** and **9** are less active, suggesting that using the phenol of triclosan to form an ether linkage has removed some of the site specific antimicrobial activity. When this information is combined with the BSA results, it seems likely that for conjugates **8** and **9** improvements in the antibacterial activity over neomycin B must therefore relate to either increased binding to cellular targets, a new resilience to enzymatic inactivation, or an increased concentration of the conjugate in the cell.

Of course, each of these options may come into play. Compounds **9**–**11** remain relatively active against MRSA, while our aminoglycoside control, neomycin sulfate has its activity reduced over

250-fold from *Staphylococcus aureus* to MRSA. The bacterial strain's resistance is likely due to the presence of neomycin-modifying enzymes that modify the drug, blocking effective binding to RNA or decreasing the drug's intracellular concentration.[5] The presence of a large hydrophobic moiety in compounds **9**–**11** likely prevents successful binding to the inactivating enzymes, leading to sustained activity. When we examine the conjugates created with the smaller chloroxylenol moiety we see that they show the expected decrease in activity against MRSA, suggesting the single aromatic ring lacks the bulk required to prevent enzyme interactions.

The increased activity of conjugates **9**–**11** against the two strains of *P. aeruginosa* is somewhat more difficult to explain. All three compounds were roughly fourfold more active than neomycin sulfate, but while *P. aeruginosa* does possess inactivating enzymes, most of its drug resistance stems from the expression of efflux pumps.[5,7] A triclosan-specific interaction is unlikely, due to the presence of a non-susceptible analogue of FabI, FabV,[26] and the inferences made by examining the activity of conjugates **8** and **9** against *E. coli* (vide supra). One possible explanation is that attaching the hydrophobic phenolics to neomycin has increased the drugs diffusion into the cell, partially overcoming the effect of the efflux pumps. This hypothesis fits well with the reduced efficacy of conjugates **9**–**11** against *P. aeruginosa* in the presence of BSA, as we would expect the hydrophobic protein to reduce the concentration of free conjugate outside of the cell, slowing diffusion and aiding efflux. The effect of efflux pumps and permeability could be further characterized using strains of *P. aeruginosa* with reduced efflux, but the relatively low activity of the conjugates may complicate matters.

In conclusion, we have produced six novel aminoglycoside–phenolic conjugates, in order to test the viability of combining known hydrophobic drugs and aminoglycosides to create compounds with an emergent activity similar to that of the cationic antibacterial peptides and cationic detergents. In general the conjugates displayed improved activity against neomycin sulfate resistant bacteria and slightly reduced activity against neomycin susceptible strains. For several conjugates activity against MRSA was found comparable to that against *S. aureus*, while activity against *P. aeruginosa* was moderately improved. Unlike previous work with analogues of CAs like cationic detergents and amphiphilic aminoglycosides,[8,11,12] our most active compounds were not appreciably hemolytic, and activity was retained in the presence of BSA. Work is currently in progress to optimize the antimicrobial activity of non-hemolytic neomycin phenol conjugates and to explore the likelihood of resistance development.

Acknowledgments

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Supplementary data

Supplementary data (experimental details) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2012.01.025.

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