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The action of norfloxacin complexes on *Tetrahymena* investigated by microcalorimetry

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Abstract $Cu(nor)_2 \cdot H_2O$ (**1**), $Zn(nor)_2 \cdot 4H_2O$ (**2**), $Ni(nor)_2 \cdot 2H_2O$ (**3**), $[Cu(nor)(phen)]NO_3 \cdot 4H_2O$ (**4**), $[Zn(nor)(phen)]NO_3 \cdot 2H_2O$ (**5**), and $[Ni(nor)(phen)]NO_3 \cdot 3H_2O$ (**6**) were synthesized and their action on *Tetrahymena* growth was studied by microcalorimetry. The growth constant (k), inhibitory ratio (I), and half-inhibiting concentration (IC_{50}) were calculated, which showed that the complexes had a strong inhibitory effect on *Tetrahymena*. All these complexes can inhibit the growth of *Tetrahymena* more strongly than norfloxacin. The norfloxacin–metal complexes exhibited better inhibitory activity than nor–phen–metal complexes. The power–time curves of *Tetrahymena* growth in the presence of norfloxacin were also measured. It was found that all complexes showed higher inhibitory activity than norfloxacin. And the inhibitory mechanism was discussed preliminarily. The diverse inhibition may be due to the ability of the complexes to penetrate into cells and the effect of these complexes on the nucleic acid. Microcalorimetry has been used extensively in many biological and chemical investigations as a universal, nondestructive, continuously running, and highly sensitive tool.

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Introduction

Norfloxacin (nor), known as fluoroquinolone, is a typical second-generation quinolone antibiotics. It is a broad-spectrum synthetic antimicrobial agent used as the clinical treatment for many infections including prostate, skin, pulmonary, digestive, and urinary tract infections [1–5]. It can effectively inhibit DNA replication by targeting the DNA gyrase, which can lead super-coils into closed-circular DNA by consuming the free energy of ATP hydrolysis [6–8].

As a N-heterocyclic compound, norfloxacin easily interacts with metal ions and forms complexes. When being the form of metallic complexes, norfloxacin posses modified pharmacological properties. Many studies showed that antibacterial activity of many norfloxacin–metal complexes is different from norfloxacin [9–11]. In addition, the studies on the interaction between quinolone–metal complexes and DNA exemplify the importance of the metal ions for the biological actions of these drugs [12, 13].

Although the interaction between quinolones and DNA undoubtedly contributes to the desired antibacterial activity, it can also be responsible, at least in part, for the toxic effects on the cell [14]. Many drugs possess pharmacological and toxicological properties when they are in the form of metallic complexes. The study of in vitro cytotoxicity of the complex (chloro)(2,2′-bipyridine)(*N*-propyl-norfloxacinato) copper(II) against two leukemia cell lines(HL-60 and K562) revealed an increased antiproliferative and necrotic effect, in comparison to the free ligand Hpr-norf [15]. Therefore, the deeper insight into the

biological properties of these complexes is important for a better understanding of their therapeutic efficacy.

1,10-Phenanthroline (phen) is one of the earliest and most extensively studied N-heterocyclic chelating agents which can form a multitude of coordination complexes with various metal ions [16–19]. Since Sigman and coworkers discovered that a mixture of phen and $CuCl_2$ can cleave DNA [20–22], the complexes of phen were variously researched. Their intercalating properties along with their function as nonradioactive nucleic acid probes and DNA cleaving agents and their biological activities such as antitumor, antibacterial, and antimicrobial have been reported [23–28].

Thus, the aim of this study was to evaluate the action of the complexes ($Cu(nor)_2 \cdot H_2O$ (**1**), $Zn(nor)_2 \cdot 4H_2O$ (**2**), $Ni(nor)_2 \cdot 2H_2O$ (**3**), $[Cu(nor)(phen)]NO_3 \cdot 4H_2O$ (**4**), $[Zn(nor)(phen)]NO_3 \cdot 2H_2O$ (**5**), and $[Ni(nor)(phen)]NO_3 \cdot 3H_2O$ (**6**)) by microcalorimetry. *Tetrahymena* are free-living ciliate protozoa that can be used as model organisms in biomedical research. So they were chosen as the biological model in this study. The growth constant (k) and half inhibitory concentration (IC_{50}) were calculated from the fundamental power–time curves of *Tetrahymena*. These parameters reflect the dynamic changes of the growth process of *Tetrahymena* under the action of the complexes, which can help to elucidate the effects of the complexes on the biological processes.

Materials and methods

Reagents

The six complexes investigated in this paper were $Cu(nor)_2 \cdot H_2O$ (**1**), $Zn(nor)_2 \cdot 4H_2O$ (**2**), $Ni(nor)_2 \cdot 2H_2O$ (**3**), $[Cu(nor)(phen)]NO_3 \cdot 4H_2O$ (**4**), $[Zn(nor)(phen)]NO_3 \cdot 2H_2O$ (**5**), and $[Ni(nor)(phen)]NO_3 \cdot 3H_2O$ (**6**). They were synthesized according to the references [29, 30]. Their structures are shown in Fig. 1.

Norfloxacin (Nor) and 1,10-phenanthroline (Phen) were obtained from Sigma. Peptone, yeast powder, and glucose were obtained from Aldrich. $Ni(NO_3)_2 \cdot 6H_2O$, $Cu(NO_3)_2 \cdot 3H_2O$, $Zn(NO_3)_2 \cdot 6H_2O$, and other common regents such as NaOH and ethanol were purchased from Sinopharm Chemical Reagent Co., China. All the chemicals used were of analytical grade and double distilled deionized water was used in all experiments.

Tetrahymena

Tetrahymena was provided by Wuhan Institute of Hydrobiology, Chinese Academy of Science, Wuhan.

The peptone culture medium (pH 7.0) contained: peptone 15 g, yeast extract 5 g, glucose 1 g, and 1000 mL double distilled deionized water. It was first sterilized in

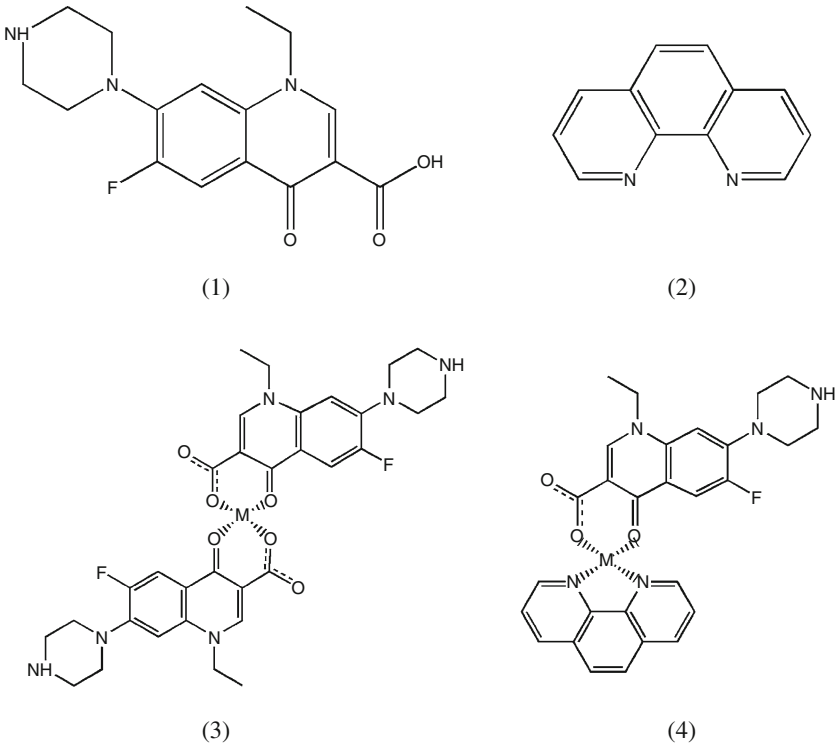

Fig. 1 The compounds: (**1**) norfloxacin (nor), (**2**) 1,10-phenanthroline (phen), (**3**) nor–metal complex, (**4**) nor–phen–metal complex

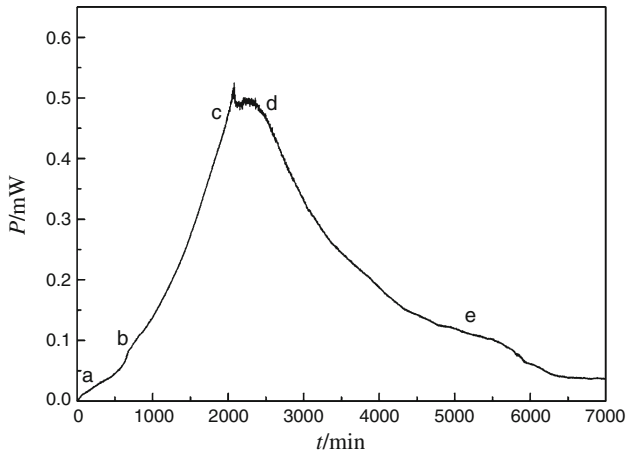

Fig. 2 The power–time curve of *Tetrahymena* growth at 28 °C

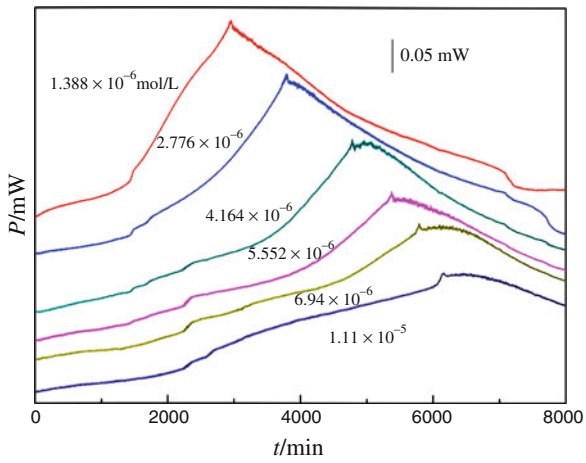

Fig. 3 The power–time curves of *Tetrahymena* in the presence of Cu–Norfloxacin complex at 28 °C

high-pressure steam at 120 °C for 30 min and then stored at 4 °C.

The cells were grown axenically without shaking at 28 °C in the peptone culture medium and maintained in exponential growth phase by reseeding.

Microcalorimetry

A TAM Air isothermal calorimeter (Thermometric AB, Sweden) was used to measure the heat production. It is an eight-channel isothermal heat conduction calorimeter operating in the milliwatt range. The thermostat was maintained at 28 °C with an absolute accuracy of 0.02 °C. The performances and the details of this instrument have been described previously [31].

The metabolic power–time curves of *Tetrahymena* were determined using ampoule method. At first *Tetrahymena* was put into the 25-mL ampoule containing 5 mL of culture medium with or without the tested complexes. Then the glass ampoules were put into the microcalorimeter. The growth of *Tetrahymena* in the absence or presence of the complexes was monitored by the microcalorimeter automatically and continuously.

Results and discussion

Power–time curves of *Tetrahymena* during growth at 28 °C

The power–time curve of *Tetrahymena* growth in culture medium at 28 °C was shown in Fig. 2. The metabolic process can be divided into four phases: the growth stagnation phase (ab), the logarithmic growth phase (bc), the stability phase (cd), and the decline phase (de).

The power–time curves of *Tetrahymena* growth affected by $Cu(nor)_2 \cdot H_2O$ (complex (**1**)) were shown in Fig. 3. As the concentration increased, the growth of *Tetrahymena* slowed down and the maximum of the power output became smaller, indicating prolongation of the whole metabolic process. However, the shapes of thermogenic curves are basically the same in the presence or absence of complexes, i.e., the four phases still existed. The curves of other complexes ((**2**), (**3**), (**4**), (**5**), and (**6**)) are similar to the result of complex (**1**).

The growth rate constant (k)

In the logarithmic growth phase the cell growth is exponential. If the cell number is n_0 at time 0, and n_t at time t, then

$$n_t = n_0 \exp(kt) \tag{1}$$

where k is the growth rate constant. If the power output of each cell is w, then

$$n_t W = n_0 W \exp(kt) \tag{2}$$

$$p_0 = n_0 W \tag{3}$$

and

$$p_t = n_t W \tag{4}$$

$$p_t = p_0 \exp(kt) \tag{5}$$

or

$$\ln p_t = \ln p_0 + kt \tag{6}$$

The thermogenic curves of the log phase growth correspond to Eqs. 5 and 6. The growth rate constant (k) is obtained by the data $\ln P_t$ and t which are taken from the curves. The obtained growth rate constants of *Tetrahymena* are listed in Table 1. It is apparent that the

Table 1 The growth rate constant (k) of *Tetrahymena* at 28 °C

Experiment number	k/min^{-1}	R^2
1	0.00148	0.9960
2	0.00140	0.9953
3	0.00134	0.9957
4	0.00125	0.9966
5	0.00127	0.9966
6	0.00120	0.9958
7	0.00131	0.9972
8	0.00143	0.9979
9	0.00136	0.9973
10	0.00142	0.9977

average growth rate constant is 0.00135 ± 0.00060 min^{-1}, showing the measurement has a good reproducibility. The correlation coefficients are very good ($R^2 \geq 0.9950$).

The growth rate constants (k) of *Tetrahymena* under the action of these complexes with different concentrations were also obtained (Table 2). When *Tetrahymena* grew in the presence of these complexes, the growth rate constants decreased with the increasing complex concentrations.

Inhibitory ratio (I) and half inhibition concentration (IC_{50})

To evaluate the toxicity of these complexes on *Tetrahymena*, inhibitory ratio is defined as:

$$I = \frac{k_0 - k_c}{k_0} \times 100\% \quad (7)$$

In the Eq. 7, k_c and k_0 are growth rate constants in the presence or absence of complexes, respectively. The results of I are also shown in Table 2.

When the inhibitory ratio is 50%, the concentration of complexes is defined as half inhibition concentration (IC_{50}). Apparently the smaller the inhibitory ratio is, the more toxic the complex is. According to the relationship between k and c, the values of IC_{50} are obtained as shown in Table 2.

Discussion

Microcalorimetry offers a powerful tool for studying the kinetics of the cell and predicting the bioactivity of medicine [32–36]. It provides kinetic and thermodynamic information that cannot be obtained by conventional bacteriological techniques, and all of this information is very significant for the studies of toxicology and pharmacology [37–41]. The power–time curves of *Tetrahymena* growth showed that the time of the log phase of *Tetrahymena* growth was prolonged with increasing concentrations of the complexes, which indicated that the *Tetrahymena* took longer time to produce a sufficient number of cells for a detectable signal and that excess complexes inhibited the growth of *Tetrahymena*. It was verified by the value of the growth rate constant. The growth rate constant k of *Tetrahymena* growth in the presence of the complexes all exhibited a concentration-dependent effect. Considering both the half inhibitory concentration and the rate constant, it could be concluded that nor–metal complexes showed stronger inhibition than nor–phen–metal complexes.

To determine whether complexes have increased inhibiting effects on *Tetrahymena* growth in comparison to free ligands, norfloxacin, the power–time curves of *Tetrahymena* growth in the presence of norfloxacin were also measured. The results of the rate constants and the inhibitory ratios were also presented in Table 2. It was found that norfloxacin had a slight inhibitory activity on the growth of *Tetrahymena* at the concentration range used to assay the bioactivity of the complexes in this study. All complexes showed higher inhibitory activity than norfloxacin. The study of Katsarou et al. [9] also showed the antimicrobial activity of the complex Cu(Hpr-norf)(Phen)Cl (MIC = 2.0 μg/mL) against *Escherichia coli* is stronger than the activity of the ligand Hpr-norf (*N*-propyl form of norfloxacin) (MIC = 4.0 μg/mL). These all indicate that the coordination of a metal ion to norfloxacin could affect the inhibition.

For metal complexes showing bioactivity, the chelate effect should be considered [9, 42, 43]. Ligands that are bound to metal ions in a bidentate fashion show higher antimicrobial efficiency toward complexes with unidentate O- or N-donor ligands. For nor–metal and nor–phen–metal complexes, norfloxacin were coordinated to metal ions through ring carbonyl and one of the carboxylate oxygens. The axial positions are occupied by two terminal nitrogen atoms of the piperazinyl residue. The chelate effect is probably the main reasons for the stronger inhibition.

The diverse inhibition shown by the nor–metal complexes and nor–phen–metal complexes may be due to the ability of the complexes to penetrate into cells. Valisena et al. [44] studied the uptake of norfloxacin by *E. coli* at different pH and monovalent/divalent metal ion concentrations. The results of the study supported a simple diffusion mechanism for norfloxacin incorporation into cells. The molecule in zwitterionic form exhibits maximum permeation properties. The uptake is strongly reduced when the drug bears a net charge as a result of ionization or complex formation with divalent ions. For nor-metal complexes, norfloxacin was in a zwitterionic state and formed simple bidentate chelating complexes [45–47], whereas nor–phen–metal complexes had net positive charge in the solution. So more nor–metal complexes could enter the cell and affect the cell growth.

Table 2 Values of k, I, and IC_{50} of *Tetrahymena* in different complexes at 28.0 °C

	$c \times 10^6$/mol L^{-1}	$k \times 10^4$/min^{-1}	R^2	I/%	IC_{50}/mol L^{-1}
Control	0	13.5	0.9977	0	–
$Cu(nor)_2 \cdot H_2O$	1.4	12.0	0.9885	11.1	3.9×10^{-6}
	2.8	7.29	0.9998	46.0	
	4.2	6.57	0.999	51.3	
	6.9	3.26	0.9913	75.9	
	11.1	1.98	0.9903	85.4	
$[Cu(nor)(phen)]NO_3 \cdot 4H_2O$	1.4	12.5	0.9928	7.4	6.1×10^{-6}
	2.8	11.4	0.9912	15.6	
	4.2	8.01	0.9928	40.7	
	7.0	6.16	0.9904	54.4	
	11.2	5.62	0.9714	58.4	
$Ni(nor)_2 \cdot 2H_2O$	1.4	13.2	0.9977	2.2	3.7×10^{-6}
	2.7	8.91	0.9936	34.0	
	4.1	5.71	0.9974	57.7	
	5.5	4.36	0.989	67.7	
	6.8	2.81	0.9814	79.2	
	8.2	2.19	0.9403	83.8	
	10.9	5.36	0.9753	86.5	
$[Ni(nor)(phen)]NO_3 \cdot 3H_2O$	1.4	8.52	0.9946	36.9	4.7×10^{-6}
	4.3	7.91	0.9897	41.4	
	5.8	3.73	0.9743	72.4	
	7.2	3.30	0.9967	75.6	
	8.7	3.21	0.9721	76.2	
	11.6	3.24	0.9745	76.0	
$Zn(nor)_2 \cdot 4H_2O$	1.3	9.81	0.9956	27.3	3.5×10^{-6}
	3.9	8.37	0.9883	54.0	
	5.2	7.71	0.9933	59.5	
	6.4	5.02	0.9918	68.4	
	7.7	3.12	0.9733	76.9	
	10.3	2.58	0.9918	80.9	
$[Zn(nor)(phen)]NO_3 \cdot 2H_2O$	1.5	9.39	0.9991	30.4	4.8×10^{-6}
	4.4	7.99	0.9995	40.8	
	5.9	3.50	0.9917	74.1	
	7.3	3.48	0.9973	74.2	
	8.8	3.48	0.9958	74.2	
	11.8	3.37	0.9957	75.0	
nor	1.7	12.8	0.9906	5.2	$>28 \times 10^{-6}$
	5.7	12.4	0.9917	8.1	
	11	9.81	0.9936	27.3	
	15	9.21	0.9978	31.8	
	17	8.96	0.9903	33.6	
	23	8.66	0.996	35.9	
	28	8.16	0.9937	39.6	

On the other hand, the effect of these complexes on the nucleic acid is another reason why these complexes showed different bioactivities. The transition metal complexes can bind to DNA via covalent mode (a labile ligand of the complexes is replaced by a nitrogen atom of DNA bases) and/or noncovalent interactions (intercalative, electrostatic, and groove binding of metal complexes along outside of DNA helix, along major or minor groove) [48].

In general, complex containing phen can insert and stack between the base pairs of DNA [49], while the norfloxacin–metal complexes can interact with DNA via noncovalent interactions comprising groove binding, electrostatic binding to phosphate group or intercalation [50]. The binding mode will influence the nucleic acids metabolism and finally inhibit the cell growth.

Conclusions

Six norfloxacin complexes were prepared. The action of these complexes on *Tetrahymena* was investigated by microcalorimetry. From the power curves, the growth rate constants (k), inhibitory ratios (I), and half inhibition concentration (IC_{50}) were calculated. Based on both the half inhibitory concentration and the growth rate constants, the action of these complexes on *Tetrahymena* was evaluated. All these complexes can inhibit the growth of *Tetrahymena*. Because of the chelate effect, all complexes exhibited higher inhibitory activity than norfloxacin. The better penetrativity of nor–metal complexes into cells and the binding mode with DNA resulted in their stronger inhibitory activity than that of nor–phen–metal complexes.

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