Cytostatic Activity of 1,10-Phenanthroline Derivatives Generated by the Clip-Phen Strategy

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The cytostatic activities of a series of twelve 1,10-phenanthroline (Phen) derivatives and of their copper complexes were studied on L1210 murine leukemia cells. Large increases in the biological activity were observed for compounds of the 3-Clip-Phen series, in which two Phen moieties were bridged at their C3 positions by an alkoxy linker, the 3-pentyl-Clip-Phen derivative showing an IC_{50} value of 130 nM while Phen shows an IC_{50} value of 2500 nM under the same conditions. IC_{50} values seemed to be modulated not only by the position, the nature, and the length of the linker of Clip-Phen but also by hydrophobicity. Since copper complexes of Phen are chemical nucleases and nucleic acids are thus a potential target for these compounds, the corresponding copper complexes were also studied. Copper complexation of the 3-Clip-Phen ligands did not increase their biological activities. Attempts to vectorize 3-Clip-Phen derivatives with a DNA binder such as spermine or with a cell-penetration peptide failed to increase their biological activity relative to the original 3-Clip-Phen series.

Introduction

The redox activity of copper complexes of 1,10-phenanthroline (Cu·Phen or $Cu(Phen)_2$) as artificial nucleases is well known.[1] These compounds have been used to footprint DNA binding molecules, to map molecular interaction, and for the preparation of sequence-specific cleaving molecules, as well as to study some oxidative DNA-cleavage mechanisms. Since $Cu(Phen)_2$ is clearly more active, we have developed Clip-Phen series containing two Phen moieties linked through tethers at their C2 or C3 positions in order to favor a stoichiometry of two phenanthroline units per copper.[2] After complexation with copper, this strategy allowed the DNA-cleavage activity to be dramatically increased in relation to that of the Phen parent compound. The nuclease activity of Clip-Phen was also successfully modulated through conjugation to different DNA binders.[3]

In parallel with these applications as biological tools, a therapeutic use of these new ligands can also be considered. The cytostatic activity of Phen itself being known,[4] we wondered whether the Clip-Phen strategy could be used to increase the potential antitumor activity of Phen derivatives. The preliminary results of these biological evaluations on leukemia cells are the topic of this publication.

Results and Discussion

The cytostatic and/or cytotoxic properties of the Phen derivatives were evaluated on a L1210 murine leukemia cell line that can be regarded as a good model for exploring the intrinsic activities of these drugs.[5] The IC_{50} values (drug concentrations inhibiting cell growth by 50% after 48 h) are presented in Table 1.

Linkage effects

We first chose to compare the cytostatic activity of Phen ($IC_{50} = 2.5$ μM) with that of the 3-Clip-Phen series (i.e., the molecules without vectorization and with two Phen units linked through their C3 positions). The 3-Clip-Phen derivatives are the most active compounds in the DNA-cleavage experiments that we have previously reported.[4] It appeared that there is no direct correlation between the nuclease activity and the IC_{50} value of the same Clip-Phen molecules. For example, 3-Clip-Phen showed no significant difference from Phen in terms of IC_{50} value (1.15 vs. 2.5 μM, respectively), whereas the copper complex of 3-Clip-Phen is the best DNA cleaver of the Phen-containing molecules. It is remarkable that 3-propyl-Clip-Phen ($IC_{50} = 0.25$ μM), the analogue of 3-Clip-Phen without the amine function in the linker, has a clearly higher biological activity than Phen itself. This significant and reproducible cytostatic activity was confirmed by a series of independent experiments performed six months after the first IC_{50} value had been measured. This experiment offered a validation of the use of the Clip-Phen strategy to increase the cytotoxicity of Phen-containing molecules.

Analogues of 3-propyl-Clip-Phen with two to five methylene groups in the linker (3-ethyl-, -propyl-, -butyl-, or -pentyl-Clip-Phen: the "3-alkyl-Clip-Phen" series) were prepared. A com-

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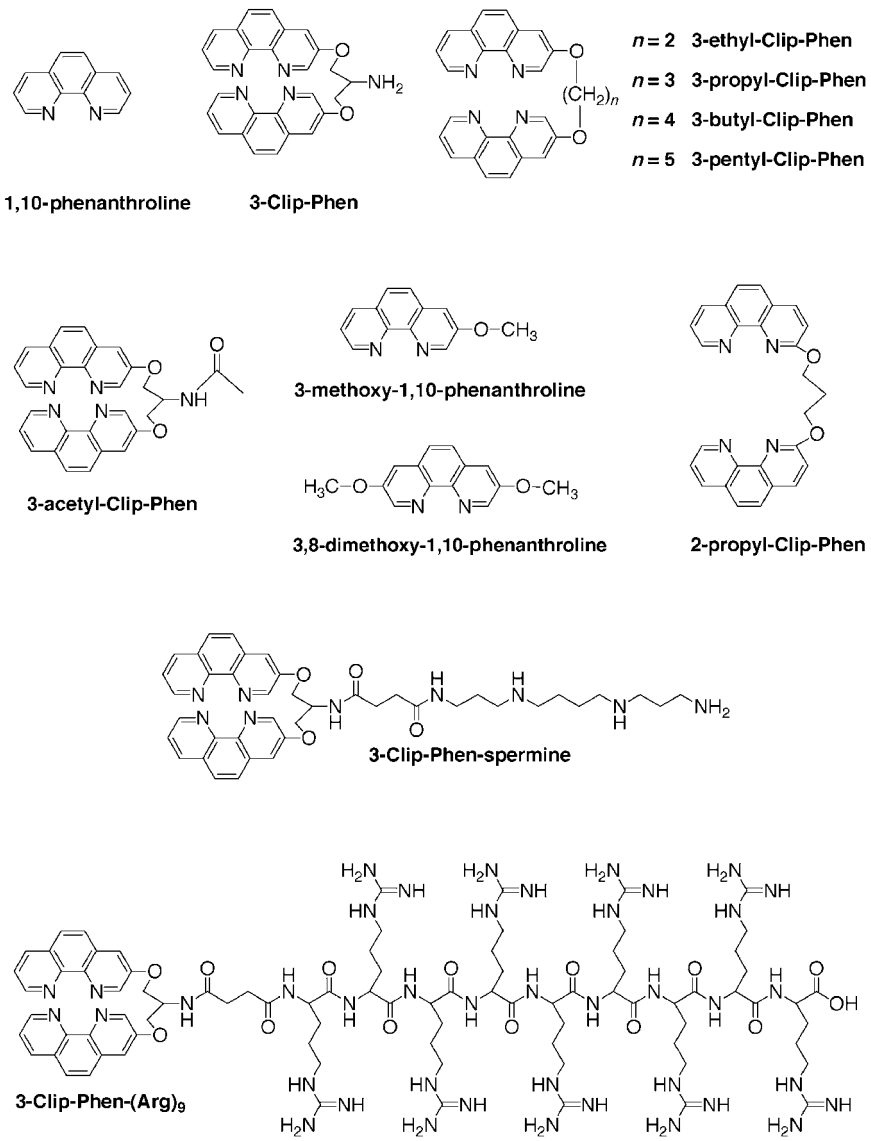


pound with a short linker, such as 3-ethyl-Clip-Phen (IC_{50} = 2.3 μM), has a cytostatic activity similar to that of Phen itself, whereas the presence of a larger number of methylene units increased the cytostatic properties. 3-Pentyl-Clip-Phen (IC_{50} = 0.13 μM), with the longer linker, is significantly more active. A relationship between the number of methylene groups in the linker and the $1/IC_{50}$ value could be established (Figure 1) and could further be useful as a parameter for the optimization of this type of compound. These 3-alkyl-Clip-Phen compounds also appeared to be more hydrophobic than Phen or 3-Clip-Phen, and an interesting relationship between calculated values ($\log P_{calc}$) and $1/IC_{50}$ values was also observed (Figure 2; P is the partition coefficient between water and octan-1-ol). However, under more physiological conditions, all these 3-alkyl-Clip-Phen compounds exhibit similar hydrophobicity (and thus lipophilicity), as determined experimentally from their distribution coefficients between octan-1-ol and phosphate buffer at pH 7.2 ($D_{7.2}$; Table 1). Similar $\log D_{7.2}$ values of around 2.8 were observed; this suggests that these Clip-Phen molecules are 500 to 700 times more soluble in octan-1-ol than in phosphate buffer at physiological values (pH 7.2).

In order to investigate whether the hydrophilic primary amine group on the linker of 3-Clip-Phen could explain its low biological activity (IC_{50} = 1.15 μM, $\log D_{7.2}$ = 2.0, $\log P_{calc}$ = 2.47), the corresponding more hydrophobic acetamido derivative 3-acetyl-Clip-Phen was prepared and evaluated (Table 1). The presence of the acetyl group on 3-acetyl-Clip-Phen did not increase the biological activity (IC_{50} = 5 μM, $\log D_{7.2}$ = 2.3, $\log P_{calc}$ = 2.80), so correlation between $\log P_{calc}$ (or $\log D_{7.2}$) and IC_{50} values in this study should be limited only to the 3-alkyl-Clip-Phen series.

In order to determine whether part of the cytostatic activity of these Clip-Phen derivatives was related to the presence of alkoxy substituents at the C3 positions of the phenanthroline units, simple phenanthroline molecules with one (3-methoxy-Phen) or two methoxy groups (3,8-dimethoxy-Phen) were prepared and evaluated. The IC_{50} values of these two molecules are 1.5 μM and 1.4 μM, respectively, in the same range as the IC_{50} value of Phen itself (IC_{50} = 2.5 μM). These data suggest that the covalent linkage of the two Phen entities effected in the Clip-Phen strategy is responsible for the increased cytostatic activity of these Clip-Phen molecules.

It should be noted that the position of attachment of the linker on the Phen units appeared to be a key parameter for modulation of the biological activities of Clip-Phen derivatives. As one example, 2-propyl-Clip-Phen, the regioisomer of 3-propyl-Clip-Phen with the linker attached at C2 positions, was almost totally inactive (IC_{50} > 100 μM). Currently we have no way to explain such a huge difference between the cytotoxicities of these two regioisomers of Clip-Phen.

Conjugation effects

The biological activities of many antitumor molecules have been efficiently increased through their conjugation to DNA binders or cell penetration agents.[6] We decided to investigate such an approach with the Clip-Phen derivatives, since we had

Table 1. *Antiproliferative activity on L1210 cell line and partition (P) or distribution (log $D_{7.2}$) coefficients.*

Compound	IC_{50} L1210 [μM] cell growth inhibition alone	with copper[a]	Partition coefficient octan-1-ol/aqueous phase log $D_{7.2}$[b]	log P_{calc}[c]
Phen	2.5	0.7	1.8	1.78 ± 0.22
3-Clip-Phen	1.15	1.65	2.0	2.47 ± 1.04
3-ethyl-Clip-Phen	2.3	2.7	2.8	3.80 ± 1.13
3-propyl-Clip-Phen	0.25	0.3	2.7	4.25 ± 1.04
3-butyl-Clip-Phen	0.17	0.23	2.8	4.68 ± 1.04
3-pentyl-Clip-Phen	0.13	0.14	2.8	5.09 ± 1.04
3-acetyl-Clip-Phen	5	10	2.3	2.80 ± 1.09
3-methoxy-Phen	1.5	1.7	2.2	2.13 ± 0.73
3,8-dimethoxy-Phen	1.4	1.6	2.4	2.47 ± 1.24
2-propyl-Clip-Phen	> 100	2	3.2	4.84 ± 0.39
3-Clip-Phen-spermine	12	21		
spermine	9	6		
3-Clip-Phen-$(Arg)_9$[d]	1.9	2.2		
$(Arg)_9$[d]	70	70		
$CuCl_2$	–	> 100		

[a] Compounds were preincubated with one equivalent $CuCl_2$ before addition to cell cultures. [b] Log values of distribution coefficient of unmetalated ligands between octan-1-ol and sodium phosphate buffer (0.1 M, pH 7.2). [c] Calculated for unmetalated ligands from www.acdlabs.com. [d] Control experiments carried out in the presence of TFA—the counterion of 3-Clip-Phen-$(Arg)_9$ and $(Arg)_9$—at 1 mM concentration showed no effect of TFA on the cell proliferation under the experimental conditions used.

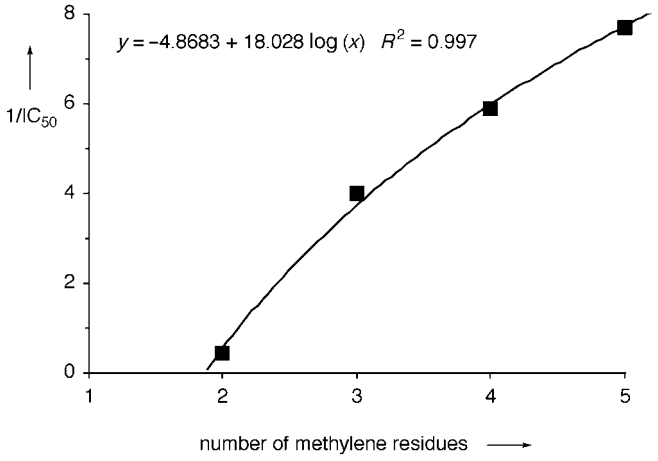


Figure 1. *Relationship between the linker lengths of 3-alkyl-Clip-Phen derivatives and the antiproliferative activity on the L1210 cell line.*

previously found these molecules to be particularly sensitive to vectorization when used as DNA cleavers.[3]

The amino group of the serinol linker of 3-Clip-Phen is perfectly adapted for making a conjugate with spermine, a natural polyamine that combines DNA binding activity and cell-penetration properties.[7] A synthetic strategy analogous to that previously optimized for the preparation of the 2-Clip-Phen-spermine isomer was used.[3] Control experiments with the copper complex of this 3-Clip-Phen-spermine ligand showed a large

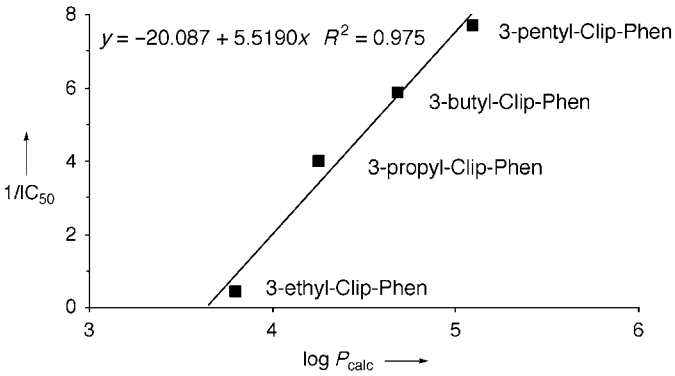


Figure 2. *Relationship between the log P_{calc} values of 3-alkyl-Clip-Phen derivatives and their antiproliferative activity on the L1210 cell line.*

increase in its nuclease activity relative to the 3-Clip-Phen complex in the classical cleaving activity evaluation on ΦX174 DNA,[2, 3a, c] confirming that the vectorization increased the affinity for DNA, a potential target (results not shown). However, the IC_{50} value observed for 3-Clip-Phen-spermine (IC_{50} = 12 μM, Table 1) is worse than that of 3-Clip-Phen itself (IC_{50} = 1.15 μM) or that of 3-acetyl-Clip-Phen (IC_{50} = 5 μM), which also has an amide function. The IC_{50} value observed for 3-Clip-Phen-spermine was in fact close to that of free spermine (IC_{50} = 9 μM; Table 1).

Cell uptake of pharmacologically active molecules has been significantly enhanced by conjugation to $(Arg)_9$, a nonapeptide derived from HIV tat protein.[8] We prepared the conjugate of 3-Clip-Phen with the unmodified $(Arg)_9$ peptide to allow the degradation of the peptide vector after cell uptake, in order to facilitate the liberation of free 3-Clip-Phen derivative inside the cells.[8] The conjugation step was performed on the solid support used during peptide synthesis in a method previously optimized for conjugation of Phen or Clip-Phen derivatives to a peptide analogue of distamycin.[3] Unfortunately, the biological activity of 3-Clip-Phen-$(Arg)_9$ (IC_{50} = 1.9 μM) was in the same range as that of 3-Clip-Phen itself (IC_{50} = 1.15 μM), indicating that this type of conjugation with a polyarginine moiety did not enhance the cytotoxicity activity of 3-Clip-Phen, at least in the cell system used for this study. Surprisingly, both 3-Clip-Phen-$(Arg)_9$ and $(Arg)_9$ showed steep slopes for the variation of their cytostatic activities as a function of the concentration difference (see Figure 3A), whereas all the other tested compounds exhibited a classical dependence for the cytostatic activity/concentration curves (see the case of 3-Clip-Phen and the corresponding copper compound in Figure 3B). For example, 1 and 5 μM of 3-Clip-Phen-$(Arg)_9$ inhibited 20 and 100% of the cell proliferation, respectively (Figure 3A). The biological mode of action of this peptide conjugate (and the corresponding copper complex) was probably different from the mode of action of the others.

These results obtained with 3-Clip-Phen conjugates indicate that the vectorization does not really increase the cytostatic activities of 3-Clip-Phen derivatives, even though their biological mode of action is probably changed.

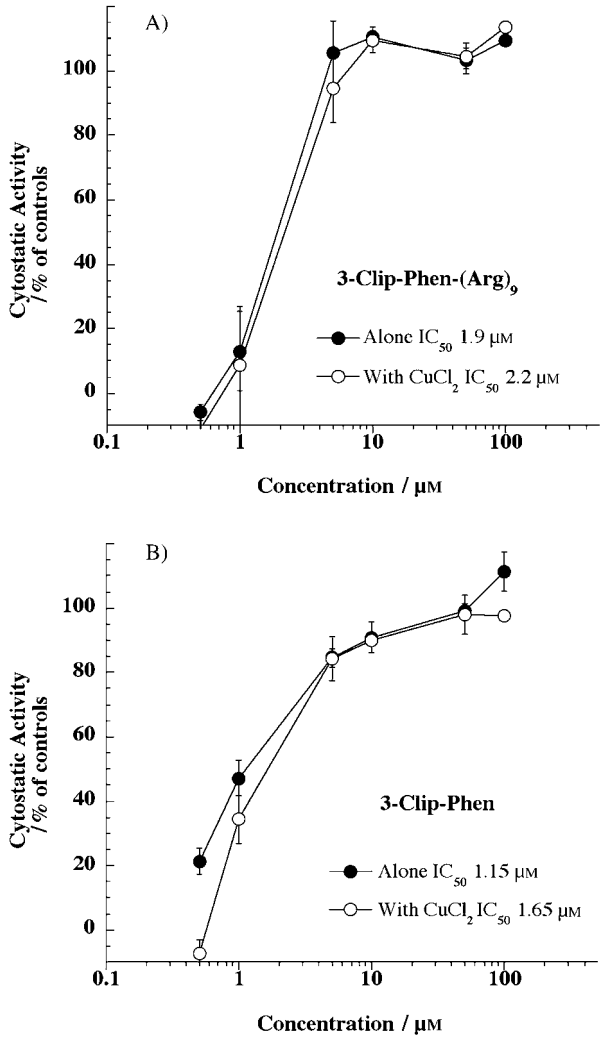


Figure 3. *Examples of cytostatic activity on L1210 cells. A) 3-Clip-Phen-$(Arg)_9$ alone (●) or after preincubation with one equivalent of $CuCl_2$ (○). B) 3-Clip-Phen alone (●) or after preincubation with one equivalent of $CuCl_2$ (○). Note the steeper slope observed in the case of A), although all compounds show similar IC_{50} values (determined at 24 h).*

Copper complexation effects

An antitumor drug such as bleomycin is active and behaves as a DNA cleaver after complexation with a redox-active metal (iron or copper) and is used therapeutically in the form of metal complexes.[9] We have previously shown that Phen-containing molecules such as Clip-Phen derivatives are obvious chelating agents and show strong nuclease activity with respect to double-stranded DNA.[3] Consequently the cytostatic activity of Cu-Clip-Phen compounds after metalation with one equivalent of $CuCl_2$ was studied.

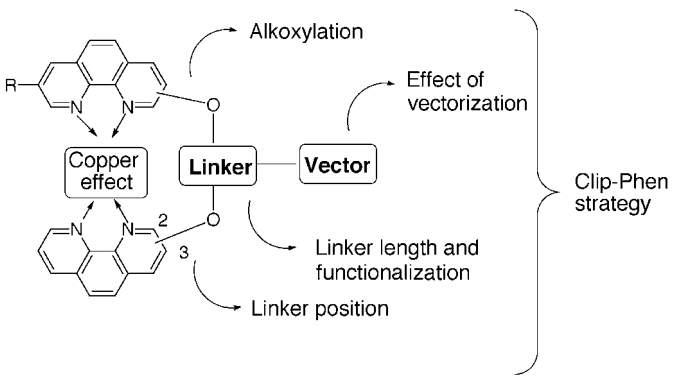
Metalation was monitored by UV-visible spectrophotometry and mass spectrometry. In the case of the Cu-3-Clip-Phen-spermine complex, absorbance typical of copper(II) complexes of 3-Clip-Phen derivatives was observed (particularly the λ_{max} at 284 nm and the metal-centered band at 745 nm, whereas λ_{max} for the unmetalated compound is at 276 nm).[2c] This suggested that it was the 3-Clip-Phen part of the conjugate that was metalated. However controls showed that unconjugated spermine also complexed copper but clearly differently (metal-centered band at 595 nm). Metalation of the 3-Clip-Phen part of 3-Clip-Phen-$(Arg)_9$ was also observed as a metal-centered band at 700 nm whereas $(Arg)_9$ in the presence of copper showed no absorbance in the visible part of the spectrum.

Whereas copper complexation of Phen moderately increased the cytostatic activity of Phen (Table 1), no significant variation in the cytostatic activities was observed in the case of the Cu-3-Clip-Phen complexes (Table 1 and Figure 3). Surprisingly, however, the cytostatic activity of 2-propyl-Clip-Phen ($IC_{50} >$ 100 μM for the free ligand) was greatly increased after copper complexation ($IC_{50} = 2$ μM); this again showed that a minor variation such as the linker attachment position can induce large variation in the biological properties of Clip-Phen derivatives.

The encouraging data obtained with 3-alkyl-Clip-Phen ligands, in comparison with the quasi-absence of activity of the C2 regioisomers, represent a strong incentive to elucidate the mechanism of action and the pharmacological target(s) of these rather simple molecules with no chiral centers.

Conclusion

Some 3-Clip-Phen derivatives exhibit cytostatic activities (IC_{50} values) on leukemia cells ranging from 130 nM to 250 nM, significantly below the threshold of Phen itself ($IC_{50} = 2.5$ μM). These preliminary biological data support the relevance of the Clip-Phen strategy that we adopted a few years ago in order to develop the biological activity of Phen-containing molecules. Comparison of IC_{50} values obtained with different Clip-Phen derivatives indicates that the biological activity is strongly modulated by structural parameters (Scheme 1). The position of attachment of the linker and its chemical composition appeared to be particularly important. Significant cytostatic activity was observed for 3-pentyl-Clip-Phen, an easily prepared molecule (only two synthetic steps are necessary for its preparation). This molecule is a good candidate for further pharmacological and toxicological in vivo studies and for investigations to identify its pharmacological target.



Scheme 1. *Structure–activity relationship parameters defined during this study.*

Experimental Section

General remarks: 3-Clip-Phen,[2a] 3-Clip-Phen-COOH,[3b] 3-acetyl-Clip-Phen,[2c] 3-ethyl-Clip-Phen,[2c] 3-propyl-Clip-Phen,[10] 3-butyl-Clip-Phen,[2c] 3-pentyl-Clip-Phen,[2c] 2-propyl-Clip-Phen,[2c] 3-methoxy-1,10-phenanthroline,[10] and 3,8-dimethoxy-1,10-phenanthroline[10] were synthesized by literature procedures.

Proton NMR spectra were recorded on a Bruker 250 MHz instrument. The ESI-MS spectrometer used was a Perkin–Elmer SCIEX API 365 in positive mode; samples were introduced into the electrospray source with a Harvard Apparatus syringe pump. UV/Vis spectra were recorded on a Hewlett–Packard 8452 A diode array spectrophotometer. DMF was dried over 4 Å molecular sieves. Other commercially available reagents and solvents were purchased from standard chemical suppliers and were used without further purification.

3-Clip-Phen-spermine: *N*-Methylmorpholine (0.55 mL, 5.0 mmol), benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (133 mg, 0.3 mmol), and 1-hydroxybenzotriazole (73 mg, 0.54 mmol) were added to 3-Clip-Phen-COOH (55 mg, 0.1 mmol) in dry DMF (11 mL). After 30 min at 50 °C, this mixture was added over 20 min to a solution of spermine (412 mg, 2.12 mmol) in water (11 mL) and stirred at 55 °C for 15 h. The reaction mixture was diluted with water (14 mL) and extracted with CH_2Cl_2 (3×25 mL). The organic phase was dried on sodium sulfate and the volume was reduced prior to precipitation of 3-Clip-Phen-spermine with diethyl ether (15 h at −10 °C) as a pale brown powder (26 mg, 36 μmol, 20%). ^{1}H NMR (250 MHz, [D$_6$]DMSO): δ = 1.59 (m, 4H), 1.78 (m, 4H), 2.50 (m, 4H), 3.24 (m, 2H), 2.6–3.0 (m, 10H), 4.60 (d, ^3J(H,H) = 5 Hz, 4H), 4.84 (m, 1H), 7.80 (dd, ^3J(H,H) = 8, 5 Hz, 2H), 7.96 (t, ^3J(H,H) = 5 Hz, 1H), 8.01 and 8.06 (AB, ^3J(H,H) = 9 Hz, 2×2H), 8.16 (d, ^4J(H,H) = 3 Hz, 2H), 8.44 (d, ^3J(H,H) = 5 Hz, 1H), 8.54 (dd, ^3J(H,H) = 8 Hz, ^4J(H,H) = 2 Hz, 2H), 8.99 (d, ^4J(H,H) = 3 Hz, 2H), 9.16 ppm (dd, ^3J(H,H) = 5 Hz, ^4J(H,H) = 2 Hz, 2H); UV/Vis (DMSO): λ (ε) = 238 (62500), 274 (44300), 294 (sh, 25300), 328 (7400), 342 nm (4800 $M^{-1}cm^{-1}$); MS (ES, positive mode): *m/z* = 732.5 $[M+H]^+$.

3-Clip-Phen-(Arg)$_9$: The nonapeptide was synthesized by solid-phase techniques by use of commercially available Fmoc amino acids. The coupling reaction with the Clip-Phen moiety was also conducted on solid phase on a 0.05 mmol scale (128 mg of FmocR_1···R_9-HMP, 1 equiv). 3-Clip-Phen-COOH (137 mg, 0.25 mmol, 5 equiv) was activated with 1-hydroxy-7-azabenzotriazole (34 mg, 0.25 mmol, 5 equiv)/diisopropylcarbodiimide (32 mg, 0.25 mmol, 5 equiv) in DMF (1 mL). This mixture was added to the resin once the Fmoc protecting group on the N-end had been removed by TFA and shaking for 15 h at room temperature. After cleavage of the solid support and deprotection, the product was purified by reversed-phase HPLC (aquapore C8 RP column (10×220 mm), A = H_2O, 0.1% TFA; B = 25% H_2O, 75% CH_3CN, 0.1% TFA; 5 min 15% B isocratic conditions, then linear gradient to 40% B over 40 min). The chromatogram was monitored at 214 nm, and the retention time of the product was 26 min. It was then lyophilized to give 3-Clip-Phen-(Arg)$_9$·11 TFA (72 mg, 0.022 mmol) as an orange solid. The purity of the product was estimated as over 94% by HPLC analysis. UV/Vis (H_2O): λ (ε) = 204 (77700), 232 (73700), 272 (42600), 296 (sh, 19800), 330 (4600), 344 nm (3100 $M^{-1}cm^{-1}$); MS (ES, positive mode) *m/z* = 1953.1 $[M+H]^+$, and major peaks observed at: 841 $[M+3H+5TFA]^{3+}$, 603.1 $[M+4H+4TFA]^{4+}$, 482.6 $[M+5H+4TFA]^{5+}$.

In vitro screening: L1210 cells (mouse leukemia, ATCC CCL219), were cultivated in DMEM (Dulbecco's MEM) supplemented with 10% fetal calf serum. Exponentially growing cells were seeded in microwell plates (24×1 mL) at 10^5 cells per well and incubated for 24 h. After that time, the cell density was approximately 3×10^5 cells per well and tested compounds were added in triplicate at various concentrations as water or DMSO solutions to a maximum percentage of 0.5% DMSO per well (day 1). Control wells received 0.5% DMSO only. After a further 24 h of incubation (day 2), cells were counted with a Coulter-Counter ZM instrument (Coultronics Inc.). The dose inhibiting the growth by 50% (IC_{50} value) was interpolated from regression curves obtained with experimental points without significant toxicity. The toxicity was determined from the decrease in the number of cells at day 2 in relation to day 1.

All incubations were carried out at 37 °C in a water-jacketed CO_2 incubator (5% CO_2, 100% relative humidity).

For experiments in the presence of copper, stock solutions of ligand (200 μL, 10 mM) were metalated by addition of $CuCl_2$ (1 equiv, 2 μL of a solution of $CuCl_2$ (1 M) were added). They were added to cell cultures after 2 h.

Copper complexes characterization: Controls showed that the free ligands and the copper complexes of Phen, 3-Clip-Phen derivatives, and 2-propyl-Clip-Phen showed the same absorbance in DMSO (used here) as in the methanol or water previously used for their characterization.[2c] For these compounds, metalation was thus monitored by spectrophotometry.

Characteristics of other compounds not published before are:

3-Methoxy-1,10-phenanthroline:[10] UV/Vis (DMSO): λ (ε) = 272 (27200), 294 (sh, 13400), 304 (sh, 8600), 314nm (sh, 3700),329 (3000), 344 nm (2300 $M^{-1}cm^{-1}$).

3-Methoxy-1,10-phenanthroline·CuCl$_2$: UV/Vis (DMSO): λ (ε) = 284 (26900), 325 (sh, 9300), 333 (sh, 7900), 347 (sh, 3400), 750 nm (70 $M^{-1}cm^{-1}$); MS (ES, positive mode): *m/z* = 308.2 (monocation, $[M-Cl]^+$), 273.0 (cuprous complex); complexes of the $[(3\text{-methoxy-1,10-phenanthroline})_2Cu]^+$ (*m/z* = 483.6) and $[(3\text{-methoxy-1,10-phenanthroline})_2CuCl]^+$ (*m/z* = 510.3) types were also observed in the spectrum.

3,8-Dimethoxy-1,10-phenanthroline:[10] UV/Vis (DMSO): λ (ε) = 280 (21600), 299 (sh, 16100), 312 (sh, 11700), 324 nm (sh, 4600), 339 (4800), 356 nm (5000 $M^{-1}cm^{-1}$).

3,8-Dimethoxy-1,10-phenanthroline·CuCl$_2$: UV/Vis (DMSO): λ (ε) = 290 (24000), 339 (sh, 12000), 360 (sh, 7000), 750 nm (30 $M^{-1}cm^{-1}$); MS (ES, positive mode): *m/z* = 338.2 (monocation, $[M-Cl]^+$); complexes of the $[(3,8\text{-dimethoxy-1,10-phenanthroline})_2Cu]^+$ (*m/z* = 543.4) and $[(3,8\text{-dimethoxy-1,10-phenanthroline})_2CuCl]^+$ (*m/z* = 572.4) types were also observed in the spectrum.

3-Clip-Phen-spermine·CuCl$_2$: UV/Vis (DMSO): λ (ε) = 284 (42700), 324 (sh, 14600), 332 (sh, 12400), 347 (sh, 6100), 745 nm (80 $M^{-1}cm^{-1}$); MS (ES, positive mode): *m/z* = 794 (cuprous complex).

Spermine·CuCl$_2$: UV/Vis (DMSO): λ (ε) = 595 nm (170 $M^{-1}cm^{-1}$); MS (ES, positive mode): *m/z* = 456.4 $[M-Cl+2DMSO]^+$, 378.3 $[M-Cl+DMSO]^+$, 300.3 $[M-Cl]^+$, 264.3 $[M-2\times Cl+H]^+$.

3-Clip-Phen-(Arg)$_9$·CuCl$_2$: (ES, positive mode): *m/z* = 2015.65 (cuprous complex), 1008.55 $[M-2Cl]^{2+}$, 672.45 $[M-2Cl+H]^{3+}$.

Metalation of the 3-Clip-Phen part of 3-Clip-Phen-(Arg)$_9$ was also observed as a metal-centered band at 700 nm, while (Arg)$_9$ in the presence of copper showed no absorbance in the visible part of the spectrum. Electrospray mass analysis of (Arg)$_9$ in the presence of one equivalent of $CuCl_2$ showed unmetalated product as major

peaks, but traces of mono- and dimetalated products were observable as minor peaks.

Log $D_{7.2}$ determination: The method was adapted from Zhang et al.[11] Ligand (2.5 mg) was dissolved in octan-1-ol (2 mL), and sodium phosphate buffer (2 mL, 0.1 M, pH 7.2) was then added. After a 3 min vortex at room temperature, followed by centrifugation for 5 min at 12000 rpm, the concentration of ligand in each layer was determined by UV/Vis spectroscopy. Samples from octan-1-ol layer were repartioned until consistent partition coefficient values were obtained. The measurement was carried out in duplicate.

Log P_{calc} determination: Log P_{calc} determination were obtained from www.acdlabs.com.

Keywords: antitumor agents · copper · cytostatic agents · DNA · phenanthroline

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