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Metallo-supramolecular complexes enantioselectively eradicate cancer stem cells in vivo

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KEYWORDS :cancer stem cell • telomerase • G-quadruplex DNA • chiral recognition • supramolecular complex

ABSTRACT: Cancer stem cells (CSCs) are responsible for drug resistance, metastasis and recurrence of cancers. However, there is still no clinically approved drug that can effectively eradicate CSCs. Thus, it is crucial and important to develop specific CSC-targeting agents. Chiral molecular recognition of DNA plays an important role in rational drug design. Among them, polymorphic telomeric G-quadruplex DNA has received much attention due to its significant roles in telomerase activity and chromosome stability. Herein, we find that one enantiomer of zinc-finger-like chiral metallohelices, $[Ni_2L_3]^{4+}$-P, a telomeric G-quadruplex-targeting ligand, can preferentially reduce cell growth in breast CSCs compared to the bulk cancer cells. In contrast, its enantiomer, $[Ni_2L_3]^{4+}$-M, has little effect on both populations. Further studies indicate that $[Ni_2L_3]^{4+}$-P can repress CSC properties and induce apoptosis in breast CSCs. This is different to the bulk cancer cells. The inhibition of breast CSC traits is involved in the nuclear translocation of hTERT. The apoptosis is associated with the induction of telomere uncapping, telomere DNA damage and the degradation of 3′-overhang. Moreover, $[Ni_2L_3]^{4+}$-P, but not $[Ni_2L_3]^{4+}$-M, has the ability to reduce tumourigenesis of breast CSCs *in vivo*. To our knowledge, this is the first report that chiral complexes show significant enantio-selectivity on eradicating CSCs.

INTRODUCTION

Cancer recurrence is closely related to the presence of cancer stem cells (CSCs), a rare cell subpopulation capable of self-renewal and differentiation.[1] Tumors which have larger proportions of CSCs compared with well differentiated tumors are linked to the lowest life expectancy.[2] CSCs also play a key role in the distant metastasis of cancer cells. Indeed, clinical studies have shown that metastatic tumors possess much greater proportions of CSCs than the primary tumors.[3] Conventional therapeutic approaches such as chemotherapy and radiotherapy effectively remove the bulk of cancer cells, but are unable to eliminate CSCs due to specific resistance mechanisms. Surviving CSCs differentiate and regenerate new tumor cells, causing tumor recurrence and metastases.[4] To improve patient survival, treatments must be capable of eliminating the entire population of cancer cells, especially CSCs. Although many potential anti-CSC agents aimed at various targets, such as multiple kinases, certain organelles and vulnerable microenvironments, have been identified, they often cause severe side effects.[5-8] Therefore, there is an urgent need to find more effective targets to discover novel anti-CSC compounds with little effects on normal somatic cells.

Telomeres have attracted much attention for anticancer therapy, as the maintenance of telomeres is required for cancer cell immortality. Telomeres are distinct nucleotide sequences at chromosome ends that protect chromosome structural integrity from degradation, illegitimate recombination and fusion.[9] Human telomere DNA includes a duplex region (2–15 kb) containing long arrays of tandem TTAGGG repeats and a single-stranded overhang (50–400 nucleotides) at the 3′ end of the G-rich strand.[10] Telomeric DNA has been found to loop back to form a T-loop structure, in which the 3′-overhang inserts the double-stranded region to form a D-loop structure which can stabilize the T-loop.[11,12] A specialized protein complex including TRF1, POT1, TRF2, RAP1, TIN2 and TPP1, called shelterin, binds and protects the 3′-overhang and maintains telomere length and structure.[13] Telomere deprotection due to the progressive telomere shortening or telomere damage results in cell growth arrest and apoptosis.[14,15] The predominant mechanism of telomere maintenance depends on telomerase, a reverse transcriptase which can add TTAGGG repeats to the ends of telomeres.[16] Telomerase is highly expressed in CSCs and most types of cancer cells but not in normal somatic cells.[17,18] Intriguingly, it has been demonstrated that CSCs are more sensitive to telomerase inhibitors than bulk cancer cells.[19,20] Therefore, targeting telomerase and telomere represents a promising anticancer therapeutic strategy that has the ability to preferentially deplete CSCs with little effects on normal cells.

Traditional telomerase inhibitors need a long lag period to induce cell senescence and apoptosis, because telomere shorten depends on cell proliferation and DNA replication.[21,22] Furthermore, alternative lengthening of telomeres (ALT) might be activated when the telomerase activity is inhibited in cancer cells, which is mediated by recombination and is one of the major limitations for the clinical application of telomerase inhibitors.[23,24] Previous reports have shown that telomere DNA cannot be elongated by telomerase when the 3′-overhang forms G-quadruplex (G4) structure.[25] Thus, the use of compounds to induce and stabilize telomere G-quadruplex structure has become a promising strategy for anticancer agent development.[26,27] This strategy has been proved effective to

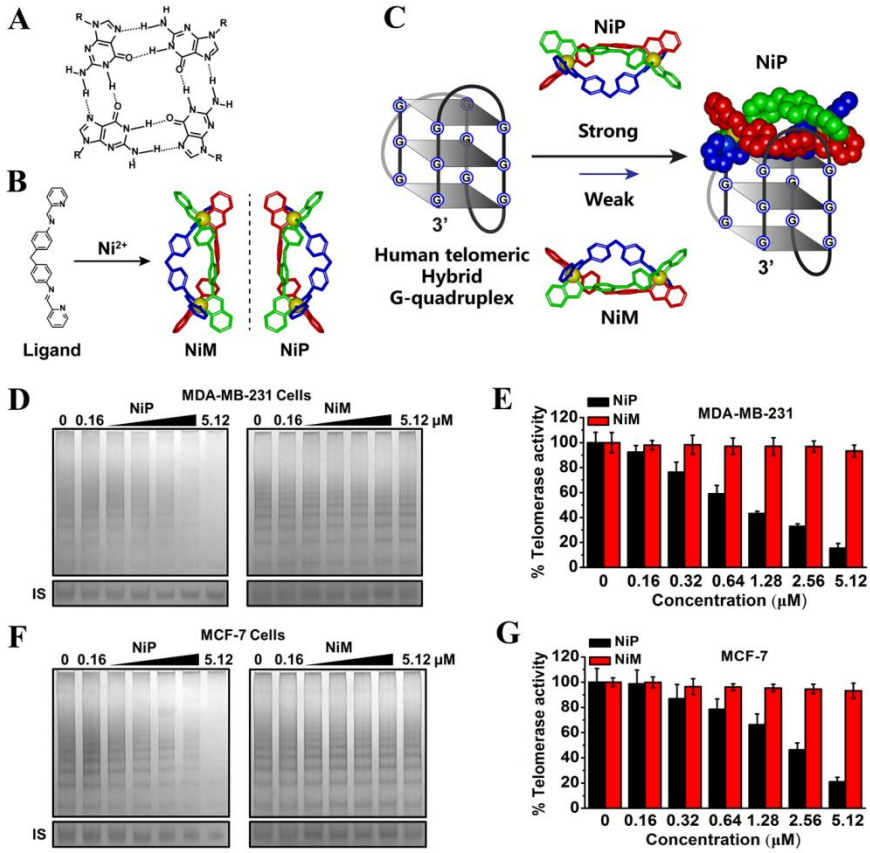

Figure 1. NiP, but not NiM, inhibits telomerase activity in breast CSCs. (A) G-quartet of guanine residues linked by Hoogsteen hydrogen bonds. (B) Structures of the M-enantiomer and P-enantiomer of $[Ni_2L_3]^{4+}$ cation. (C) Representative illustration of NiP selectively recognizing of human telomere G4 DNA. (D, F) Inhibition of telomerase activity mediated by NiP in MDA-MB-231 and MCF-7 mammospheres. Mammospheres were exposed to different concentrations of NiP and NiM for 7 days, then standard TRAP assay was performed with equivalent amounts of protein. (E, G) Quantification of telomerase activity was performed as the percent of the control sample without treatment of NiP and NiM. The results were shown as the means ± SD.

shorten telomeres and to perturb telomere function directly, which would result in short-term DNA damage and apoptosis in cancer cells.[28,29] A large number of G4 ligands have been designed and synthesized in recent years to perturb telomerase and telomere function for cancer treatment.[30-38] However, few G4 ligands exhibit high selectivity towards telomere G4 DNA. Chiral molecular recognition of DNA has attracted much attention because of its important application in rational drug design.[39,40] Human telomere G4 DNA (Figure 1A) is polymorphic, and its structural transition is associated with many important life events.[26,41,42] Chiral recognition of telomeric G4 can offer a novel strategy for development of G4 selective ligands. Chirality is closely associated with the specificity and binding activity of ligands to G4. Thus, targeting of telomeric G4 DNA by chiral compounds can identify highly selective agents which are more specific and effective to inhibit telomerase activity and disturb telomere structure in CSCs. We have recently reported that one enantiomer of chiral metallo-helices, $[Ni_2L_3]^{4+}$-P (NiP; see structure in Figure 1B) is able to selectively recognize and stabilize telomeric G4 (Figure 1C) and inhibit telomerase activity.[43,44]

Herein, we investigate the effects of chiral supramolecular complexes (NiP and NiM) on breast CSCs, and find that NiP, rather than NiM, preferentially inhibits cell growth in breast CSCs compared to the bulk cancer cells. Intriguingly, NiP has remarkable different roles in breast CSCs and bulk cancer cells. NiP inhibits breast CSC properties, induces breast CSC apoptosis and has little effect on senescence, whereas NiP promotes cell apoptosis and senescence in the bulk cancer cells.[45] Further studies reveal that telomere uncapping with the delocalization of TRF2 and POT1 from telomeres, telomere DNA damage and the degradation of 3′-overhang result in the apoptosis of breast CSCs. The reduction of CSC properties is associated with the nuclear translocation of hTERT. Moreover, NiP has the ability to reduce tumourigenesis of breast CSCs *in vivo*. Overall, our data indicates that the enantiomer NiP can effectively deplete breast CSCs *in vitro* and *in vivo*. To the best of our knowledge, this is the first example that chiral complexes show contrasting enantio-selectivity on eradicating CSCs. Our work will shed light on the application of chiral agents in anti-CSC therapy.

RESULTS

For verifying the effects of chiral metallo-supramolecular complexes (NiP and NiM) on CSCs, MDA-MB-231 and MCF-7 cell lines containing inherent breast CSC subpopulation were used. These two types of cells were cultured in anchorage-independent and serum-free culture condition to form mammospheres, which grew robustly from single cell and enriched with breast CSCs.[46] The primary mammosphere formation assay showed that MDA-MB-231 and MCF-7 cells contained a CSC population of 0.39% and 1.04% respectively (Figure S1A-B). Using $CD44^+/CD24^{-/low}$

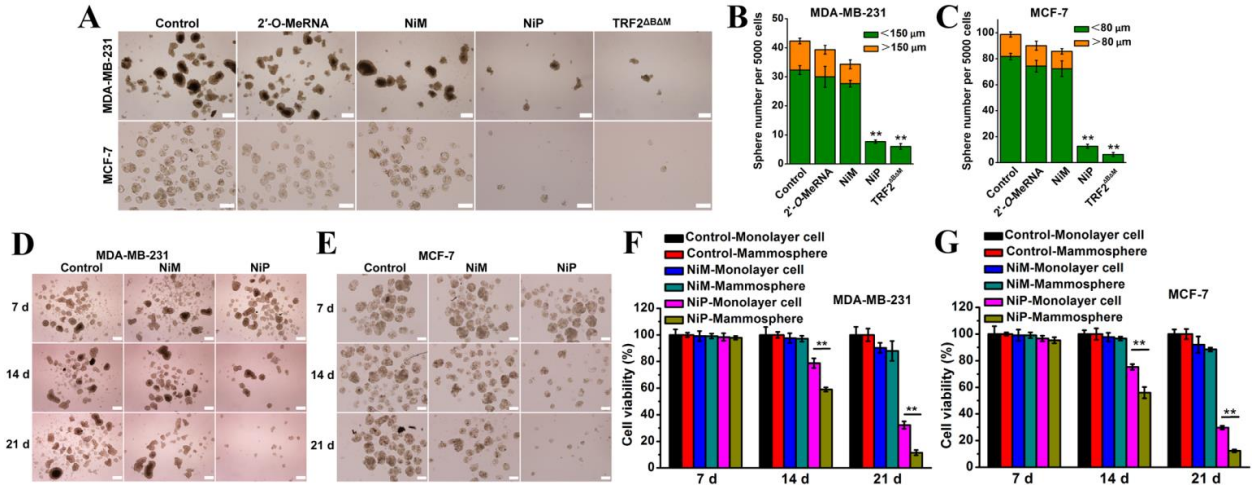

Figure 2. NiP, but not NiM, eliminates breast CSCs. (A) Reduction of breast CSC proportion induced by NiP in MDA-MB-231 and MCF-7 monolayer cells. MDA-MB-231 and MCF-7 cells were treated with NiP or NiM (5.12 μM), or transfected with 2′-*O*-MeRNA and TRF2$^{\Delta B\Delta M}$ for 3 weeks, then 5000 cells were cultured in CSC medium for 7 d to form mammospheres. The mammosphere formation was determined by microscopic examination. Scale bar = 100 μm. (B, C) The mammospheres were quantitated. (D, E) Inhibition of mammosphere formation induced by NiP in serial passaging of breast CSCs. The secondary mammospheres of MDA-MB-231 and MCF-7 cells were incubated with NiP or NiM and subcultured every 7 d for 3 weeks. The mammosphere formation was determined by microscopic examination. Scale bar equals 100 μm. (F, G) MDA-MB-231 and MCF-7 cells were cultured in ultra-low attachment plates or adherent monolayer culture, and treated with NiP or NiM (5.12 μM) for 21 d. The cell viability was determined at the indicated time. The results were shown as the means ± SD of three separate experiments. $^{**}P < 0.01$.

as breast CSC surface markers,[47-49] we observed that the percentage of breast CSC population increased to 96.7% and 98.3% respectively in the secondary spheres (Figure S1C). Typical microscopy images of the mammospheres at three different passages showed that there was no apparent change in the morphology of the mammospheres with serial passaging (Figure S1D), suggesting that our method was effective for breast CSC enrichment.

We first examined the effects of the two enantiomers on telomerase activity. The telomerase was prepared from the secondary mammospheres and subjected to a modified TRAP-G4 assay as described previously.[50] As shown in Figure S2A-B, telomerase activity was inhibited by NiP in a concentration-dependent manner and almost complete inhibition was observed at the concentration of 320 nM. The IC_{50} value for NiP on telomerase inhibition was 73.14 ± 2.23 nM. Furthermore, these concentrations of NiP had no inhibitory effect on telomerase substrate internal control. Moreover, slight inhibition of telomerase activity was found in the group treated with NiM, indicating that the two enantiomers had chiral selectivity on inhibition of telomerase activity.

We further investigated whether there was a difference between the two enantiomers in telomerase inhibition in living breast CSCs. MDA-MB-231 and MCF-7 mammospheres were exposed to NiP and NiM at concentrations ranging from 0.16 to 5.12 μM for 7 days. Then telomerase activity was tested with a conventional TRAP assay.[43] As shown in Figure 1D-G, treatment of NiP induced a concentration-dependent inhibition of telomerase activity in breast CSCs. The IC_{50} values of NiP on telomerase inhibition were 1.08 ± 0.10 and 2.03 ± 0.09 μM in MDA-MB-231 and MCF-7 mammospheres, respectively. In contrast, only slight inhibition of telomerase activity was observed in the cells treated with NiM. Taken together, NiP, but not NiM, had the ability to inhibit telomerase activity in breast CSCs. We also synthesized the methyl-substituted (at 3'- and 5'-position) supramolecular compounds $[Ni_2L^3_3]^{4+}$ and $[Ni_2L^5_3]^{4+}$ (NiP-3, NiM-3, NiP-5 and NiM-5),[51,52] and tested their effects on telomerase activity in MDA-MB-231 mammospheres (Figure S3-4). The inhibitory effects of these derivatives on telomerase were less than NiP (the IC_{50} value of NiP-3 and NiP-5 on telomerase inhibition were 4.34 ± 0.24 and 1.21 ± 0.12 μM, respectively).

Telomerase inhibition associates with growth suppression, so we assessed whether NiP could deplete CSC proportion in MDA-MB-231 and MCF-7 monolayer cells. In this assay, 5.12 μM was used because this concentration of NiP induced almost complete inhibition on telomerase activity but had no acute toxicity on breast CSCs (Figure S5A-B). MDA-MB-231 and MCF-7 cells were exposed to NiP and NiM for 3 weeks, and then the cells were cultured in CSC medium for 7 d to form mammospheres. As shown in Figure 2A-C, NiP treatment inhibited the formation of mammospheres in MDA-MB-231 and MCF-7 cells, especially, no larger mammosphere (> 150 μm in MDA-MB-231 cells and > 80 μm in MCF-7 cells) was observed in the cells treated with NiP, indicating that NiP was able to deplete breast CSCs from bulk tumor populations in these two cell lines. For clarifying the inhibition of mammosphere formation mediated by NiP was dependent on telomerase activity or telomere structure, MDA-MB-231 and MCF-7 cells transfected with 2′-*O*-MeRNA (an inhibitor of telomerase activity)[53] or transfected with TRF2$^{\Delta B\Delta M}$ mutation, which would lead to dysfunctional telomere,[54] were also subjected to mammosphere formation assay. Transfection of 2′-*O*-MeRNA did not inhibit mammosphere formation in both MDA-MB-231 and MCF-7 cells, however, obvious inhibition was observed in the cells transfected with TRF2$^{\Delta B\Delta M}$ (Figure 2A-C), this was similar to the cells treated with NiP, indicating that mammosphere formation inhibition caused by NiP was dependent on telomere structure, but not telomerase activity. The expression of TRF2$^{\Delta B\Delta M}$ in MDA-MB-231 and MCF-7 cells was detected by western blot assay targeting Myc-tag

(Figure S6A-C), furthermore, transfection of

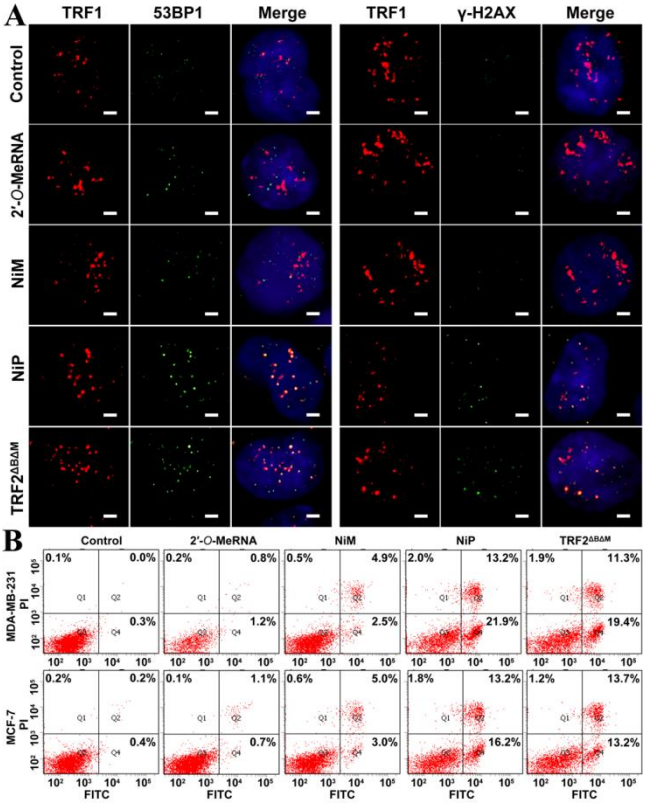

Figure 3. NiP induces telomere DNA damage and apoptosis. (A) Representative confocal images of merged 53BP1 (green)/TRF1 (red) or γ-H2AX (green)/TRF1 (red). MDA-MB-231 mammospheres were treated with NiP and NiM (5.12 μM), or transfected with 2′-*O*-MeRNA and TRF2$^{\Delta B\Delta M}$ for 2 weeks, then the cells were stained with the antibodies against 53BP1 (green)/TRF1 (red) or γ-H2AX (green)/TRF1 (red) and DAPI (blue). Scale bar equals 5 μm. (B) Cell apoptosis was detected by Annexin V-FITC/PI double staining in MCF-7 and MDA-MB-231 mammospheres exposed to NiP or NiM (5.12 μM), or transfected with 2′-*O*-MeRNA and TRF2$^{\Delta B\Delta M}$ for 3 weeks.

pcDNA3.1 (empty vector) did not affect mammosphere formation in both MDA-MB-231 and MCF-7 cells (Figure S6D).

We further examined the effects of NiP and NiM on mammosphere formation in serial passaging. The secondary mammospheres of MDA-MB-231 and MCF-7 cells were incubated with NiP and NiM and subcultured every 7 d for 3 weeks. As shown in Figure 2D-E, mammosphere formation was significantly inhibited after treatment with NiP for 2 weeks, followed by almost complete inhibition after additional 1 week, suggesting that NiP could eliminate breast CSCs in serial passaging. In contrast, there was no evident effect on mammosphere formation in the cells treated with NiM.

To test whether NiP could predominantly inhibit cell growth in breast CSCs, the sensitivity of monolayer cells and mammospheres to NiP was examined. As shown in Figure 2F-G, a time-dependent inhibition of cell viability was observed in both populations after treatment with NiP, however, mammospheres were more sensitive to NiP than the cells in monolayer cultures ($P < 0.01$), indicating that NiP preferentially inhibited cell growth in breast CSCs compared with the bulk cancer cells.

It has been demonstrated that growth inhibition mediated by telomere-targeting agents often involves in DNA damage.[27] Thus, we tested whether NiP could induce DNA damage in breast CSCs. As shown in Figure S7A-B, the phosphorylation of H2AX (γ-H2AX), a common marker of DNA double-strand break,[9] was significantly increased after 2 weeks treatment with NiP (5.12 μM). Immunofluorescence results showed that 53BP1, another DNA damage response factor,[9] and γ-H2AX formed foci during NiP treatment (Figure S7C-D), confirming that NiP could induce DNA damage response. Quantitative analysis results revealed that the percent of cells containing 53BP1 and γ-H2AX foci exceeded 80% after treatment with NiP (Figure S7E-F). In contrast, the same concentration of NiM had little effect on DNA damage. The up-regulation of γ-H2AX and more 53BP1 and γ-H2AX foci were also observed in the cells transfected with TRF2$^{\Delta B\Delta M}$, but not 2′-*O*-MeRNA. Collectively, these results indicated that the growth inhibition of breast CSCs mediated by NiP involved in the induction of DNA damage.

To determine whether DNA damage response induced by NiP occurred at telomeres, double immunofluorescence experiment was performed in breast CSCs. Confocal microscopy showed that most of the 53BP1 and γ-H2AX foci induced by NiP co-localized with TRF1 (Figure 3A), forming the telomere dysfunction-induced foci (TIFs).[9,55] Quantitative results showed that the cells with more than four 53BP1/TRF1 or γ-H2AX/TRF1 co-localizations was significantly increased after NiP treatment (the percentage of TIFs-positive cells up to nearly 70% upon treatment), with an average number of about twenty TIFs per cells (Figure S8A-B). These results were further confirmed by ChIP-qPCR,[50,56] as shown in Figure S8C, the amount of 53BP1 and γ-H2AX combined with telomeres in the cells treated with NiP was obviously increased ($P < 0.01$). Telomere DNA damage induced by NiP was very potent because the percentage of TIFs-positive cells was similar to that tested in the cells transfected with TRF2$^{\Delta B\Delta M}$ (Figure S8A).[55] However, slight telomere DNA damage response was observed in the cells treated with NiM or transfected with 2′-*O*-MeRNA.

It has been reported that the formation of quadruplex structures at telomeres induced by G-quadruplex-binding ligands may lead to delocalization of telomere binding proteins, resulting in telomere uncapping and DNA damage.[56-58] Therefore, we examined the localization of TRF1, POT1 and TRF2, which are telomere binding proteins and induce telomeric DNA damage and telomere dysfunction when they dissociate from telomeres[9,54,55] in breast CSCs treated with NiP. ChIP-qPCR assay showed that NiP had little effect on the binding of TRF1 to telomeres (Figure S9A). However, significant reduction of the binding of POT1 and TRF2 was observed in the cells treated with NiP ($P < 0.01$). Confocal microscopy results also revealed that NiP markedly delocalized TRF2 and POT1 from the telomeres which were represented by TRF1 foci (Figure S9B). The percentage of cells with more than four POT1/TRF1 or TRF2/TRF1 co-localizations was reduced to less than 20% in the cells treated with NiP, similar to that tested in the cells transfected with TRF2$^{\Delta B\Delta M}$ (Figure S9C-D). Furthermore, the expression of TRF2 and POT1 did not changed (Figure S9E), indicating that the reduction of TRF2 and POT1 at telomeres induced by NiP was not due to the down-regulation of these proteins.

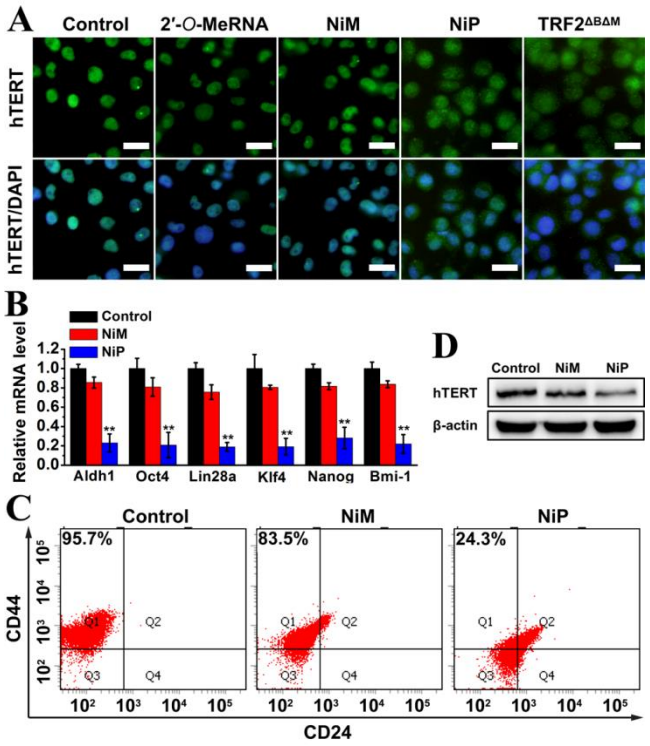

Figure 4. NiP induces translocation of hTERT from nucleus to cytoplasm and represses breast CSC properties. (A) The subcellular localization of hTERT in MDA-MB-231 mammospheres treated with NiP and NiM (5.12 μM), or transfected with 2′-*O*-MeRNA and TRF2$^{\Delta B\Delta M}$ was analyzed by staining with the antibody for hTERT. Representative images were presented. Scale bar = 20 μm. (B) qRT-PCR analysis of the expression of CSC makers (Aldh1, Oct4, Lin28a, Klf4, Nanog and Bmi1) in MDA-MB-231 mammospheres treated with NiP and NiM for 2 weeks. $^{**}P < 0.01$. (C) The proportion of CD44^{+}/CD24$^{-/low}$ cells in MDA-MB-231 mammospheres incubated with NiP and NiM was measured by flow cytometry. (D) Western blot assay of the expression of hTERT in MDA-MB-231 mammospheres treated with NiP and NiM for 2 weeks. Equal protein loading was evaluated by β-actin.

Previous studies have shown that when the telomere capping is altered, the unprotected telomeres can be accessed and labeled by terminal deoxytransferase (TdT) which can add Cy3-conjugated deoxyuridine to the naked telomere ends.[59] So we investigated TdT signals in the cells treated with NiP to confirm telomere uncapping. As shown in Figure S10A-B, most of the TdT signals (nearly 70%) co-localized with TRF1 in NiP-treated cells, indicative of robust telomere uncapping. Similar results were observed in the cells overexpressing TRF2$^{\Delta B\Delta M}$. However, little TdT-Cy3 was detectable in the cells transfected with 2′-*O*-MeRNA.

The loss of TRF2 and POT1 from telomeres can induce single-stranded G-overhang degradation.[57,60] Therefore, we measured the telomeric G-overhang length by hybridization protection assay (HPA) as described previously.[57,60] As shown in Figure S11A, the telomeric G-overhang length was significantly reduced after treatment with NiP for 2 weeks ($P < 0.01$), whereas little reduction was observed in the total length of telomeres, suggesting the depletion is specific to single-stranded G-tails. Furthermore, the degradation of G-overhang was accompanied with the emergence of micronuclei, indicative of severe genotoxicity (Figure S11B-C). These results were also obtained by transfection of TRF2$^{\Delta B\Delta M}$, but not 2′-*O*-MeRNA.

Next, we investigated whether NiP-mediated telomere dysfunction could result in senescence and apoptosis in breast CSCs. To detect cell senescence, the expression of p21 and p16 (the major markers of senescence) was examined in breast CSCs after exposure to NiP for 3 weeks. As shown in Figure S12, NiP had little effect on the expression of p21 and p16, indicating that NiP could not induce senescence. However, NiP induced significant apoptosis (35.1% in MDA-MB-231 mammospheres and 29.4% in MCF-7 mammospheres) in breast CSCs (Figure 3B). Similar results were obtained by transfection of TRF2$^{\Delta B\Delta M}$, but not 2′-*O*-MeRNA. Overall, these results indicated that NiP, but not NiM, had the ability to induce telomere uncapping and activate telomere DNA damage response, resulting in growth suppression and apoptosis in breast CSCs.

Growing evidence suggests that human telomerase reverse transcriptase (hTERT), the catalytic subunit of telomerase, can be phosphorylated at site tyrosine 707 and reversibly translocate from the nucleus to cytoplasm in response to cell stress.[61,62] To investigate whether the localization of hTERT was changed by NiP treatment, western blot and immunofluorescence assay were applied. Western blot results showed that the phosphorylation level of hTERT was significantly increased on exposure to NiP (Figure S13). Immunofluorescence results revealed that hTERT translocated to cytoplasm after NiP treatment, as observed in the cells transfected with TRF2$^{\Delta B\Delta M}$ (Figure 4A). It has been reported that hTERT is a key regulator of the stemness of CSCs, which can regulate the transcription of stemness markers, such as Oct4, Klf4 and Myc, to modulate CSC stemness.[63-65] Therefore, we tested whether the stemness of CSCs was affected by the nuclear translocation of hTERT. RT-PCR results showed that the expression of stem cell markers (Aldh1, Oct4, Lin28a, Klf4, Nanog and Bmi1) in MDA-MB-231 mammospheres treated with NiP for 2 weeks was distinctly reduced, whereas little effect was observed in NiM-treated cells (Figure S14). These results were further evidenced by qRT-PCR (Figure 4B). Flow cytometry analysis also showed that the stem cell marker CD44 was drastically reduced after treatment by NiP with the up-regulation of CD24, which resulted in significant reduction of CD44^{+}/CD24$^{-/low}$ population (Figure 4C). Meanwhile, we observed that the overall expression of hTERT was reduced after NiP treatment, whereas this effect was slight in the cells treated with NiM (Figure 4D). Collectively, these results demonstrated that NiP, but not NiM, had the ability to repressed breast CSC traits.

To evaluate the effect of NiP on tumour-initiating capacity of breast CSCs *in vivo*,[66,67] MDA-MB-231 cells were subcutaneously injected into nude mice at decreasing dilutions (5×10^5, 1×10^5 and 1×10^4 cells) and treated with NiP, NiM or PBS (control) for 4 weeks. As shown in Table 1 and Figure 5A-B, the number and size of the tumours in NiP-treated group were less than PBS- and NiM-treated groups for 5×10^5 cells injected. Furthermore, there was no tumor formation in the mice seeded with 1×10^5 cells after treatment with NiP. In contrast, significant tumor formation was observed in control and NiM-treated groups injected with the same number of cells, suggesting that NiP, but not NiM, could decrease the tumorigenic potential of breast CSCs *in vivo*. To further verify the effect of NiP on CSCs *in vivo*, primary tumour cells

Table 1. The effects of NiP and NiM on tumorigenesis *in vivo*

Treatment	Cell number	Tumor formation
Control	500 000	5/6
	100 000	3/6
	10 000	0/6
NiM	500 000	5/6
	100 000	2/6
	10 000	0/6
NiP	500 000	2/6
	100 000	0/6
	10 000	0/6

Different numbers of MDA-MB-231 cells were injected subcutaneously and mice were treated with NiP or NiM once a day. The numbers represent 'the number of mice with tumorigenesis/the number of mice in every group'.

isolated from tumor tissues were subjected to mammosphere formation assay. Single cells were seeded into ultralow adherence plates, and cultured in CSC medium for 7 d, then mammosphere formation was tested. As shown in Figure 5C-D, the number of mammospheres generated from the cells derived from NiP-treated tumours were obviously reduced compared with control group, especially, there was no larger mammosphere (> 150 μm) was observed in NiP-treated group. In contrast, the number and size of mammospheres come from the cells separated from NiM-treated tumours were similar to that in control group, suggesting that NiP rather than NiM could diminish breast CSC population *in vivo*. Moreover, no body weight drop was observed in all groups (Figure S15A). Importantly, the histologic appearance of major organs, such as heart, liver, lung, kidney and spleen, was normal in NiP-treated group (Figure S15B), indicative of the extremely low toxicity of NiP.

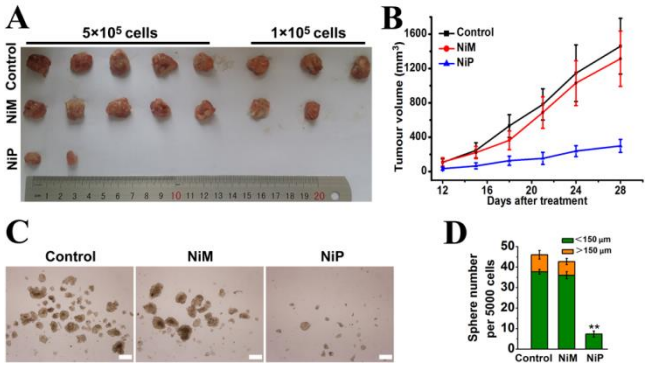

Figure 5. NiP inhibits tumour-initiating capacity of breast CSCs *in vivo*. (A) Photographs of the dissected tumors. MDA-MB-231 cells were injected subcutaneously and mice were treated with NiP or NiM once a day for 4 weeks. (B) Tumor growth curves of tumor-bearing mice after NiP or NiM treatment. (C) Mammosphere formation of the cells separated from tumor tissues treated with NiP or NiM. (D) The mammospheres were quantitated. $^{**}P < 0.01$.

DISCUSSION

Telomerase is highly expressed in CSCs and most cancer cells but not in normal somatic cells.[17,18] Thus, telomerase inhibitors have potential to remove both CSCs and bulk cancer cells, and

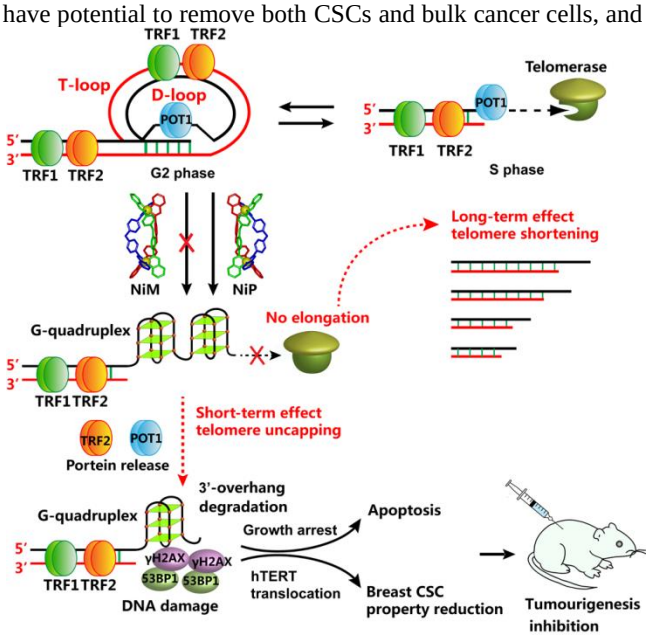

Figure 6. Schematic illustration of telomerase activity inhibition, telomere DNA damage, breast CSC property reduction, apoptosis and tumourigenesis inhibition induced by NiP in breast CSCs. Telomeric DNA is postulated to loop back to form a T-loop structure, in which 3′-overhang inserts double-stranded region to form a D-loop structure which can stabilize the T-loop. During DNA replication, the 3′-overhang can be accessed and elongated by telomerase. However, in the presence of NiP, but not NiM, the G-rich telomeric strand self-assembles into a G4 structure, leading to telomere elongation block. The persistence of G-quadruplex results in telomere uncapping, telomeric DNA damage, 3′-overhang degradation and apoptosis. Meanwhile, cell stress mediated by NiP can induce hTERT translocation from nuclear to cytoplasm, leading to the inhibition of breast CSC traits. The induction of breast CSC trait loss and apoptosis caused by NiP result in the abrogation of tumour initiation *in vivo*.

have little effect on normal cells. Moreover, it has been demonstrated that CSCs are more sensitive to telomerase inhibitors than the bulk cancer cells.[19,20] Therefore, targeting telomerase represents a promising anticancer therapeutic strategy with the ability to deplete CSCs predominantly. Telomeric G4 ligands as one type of telomerase inhibitors have great potential in the development of CSC-targeting drugs because the agents not only can inhibit telomerase activity resulting in telomere shortening in the long term but also can disturb telomere structure in the short term.[28,29] In this study, we reported that chiral metallo-supramolecular complex NiP, which displayed preferential binding to telomere G4, had the ability to induce telomerase inhibition and dissociation of telomere associated proteins from telomeres, leading to telomere DNA damage and apoptosis in breast CSCs (Figure 6). Similar results were obtained by transfection of $TRF2^{\Delta B\Delta M}$ (a mutant of TRF2), but not 2′-*O*-MeRNA (a telomerase inhibitor), demonstrating that NiP induced CSC apoptosis through destroying telomere structure before the length of telomere is shortened.

Drug development aims to discover novel therapeutic agents targeting the key enzymes, protein, nucleic acids, and the interactions of protein-nucleic acid, receptor-ligand or protein-protein to mitigate or cure relevant diseases. Almost all of the enzymes, nucleic acids and proteins are chiral, and

these biomolecules have stereoselectivity towards their binding ligands.[68] Indeed, it has been demonstrated that chirality can determine drug efficacy and safety.[69] Therefore, chiral recognition of targets is crucial for design and development of drugs. Chiral drugs have played an important role in human disease treatments. Chiral compounds account for more than 50% of currently used drugs and this ratio exhibits an increasing tendency.[68] In some cases, one enantiomer is active, while the other may show side effects, including toxicity. Thus, investigation of the effect of individual enantiomers is important for new drug discovery.[68] The structural transition of telomeric G-quadruplex DNA is associated with many significant life events. Chiral recognition of telomeric G-quadruplex in CSCs can discover highly selective agents which can specifically and effectively inhibit telomerase activity and perturb telomere function in CSCs. Targeting of telomere G4 DNA via chiral compounds may offer a novel strategy for development chiral drugs that can eliminate CSCs. Our previous studies have demonstrated that one enantiomer of chiral metallohelices, NiP, rather than NiM, has the ability to selectively stabilize telomere G4 DNA.[43,44,70] The steric configuration matching of NiP, but not NiM, with the G-quadruplex DNA plays a key role for their enantioselective interaction. When interacting with G-quadruplex DNA, NiP stacks at the end G-quartet of G-quadruplex by hydrophobic interaction. In addition, for these metal complexes, central metal ions Ni^{2+} are coordinatively saturated in octahedron geometry and hence would not coordinate directly to the DNA. Thus, the cationic charges of Ni^{2+} further favor their electrostatic interactions with G-quadruplex DNA (Figure 1C).[43,44,70] Our study reported here showed that enantiomer NiP could observably eliminate breast CSCs via inducing telomere DNA damage, breast CSC property loss and apoptosis. In contrast, enantiomer NiM had little effect on breast CSCs. To our knowledge, this is the first report that chiral compounds show enantio-selectivity in eradicating CSCs.

In addition to telomere length-dependent function, hTERT, the catalytic subunit of human telomerase, can also modulate gene expression in CSCs.[63] There is emerging evidence that hTERT can regulate the transcription of pluripotency markers, such as Oct4, Klf4 and Myc, to modulate CSC stemness.[64,65] Our results showed that hTERT translocated from the nucleus to cytoplasm after treatment with NiP, meanwhile, the expression of CSC markers (Aldh1, Oct4, Lin28a, Klf4, Nanog and Bmi1) were reduced after NiP treatment (Figure 4B). We reasoned that the reduction of hTERT in nucleus induced by cell stress could perturb the network of pluripotency transcription factors, resulting in the inhibition of CSC properties, accompanied by down-regulation of CSC markers and hTERT whose expression is closely correlated with stem cell-like properties.[71,72] The perturbation of the pluripotency transcription factor network may be one of the reasons that CSCs are more sensitive to NiP than the bulk cancer cells.

Telomeres are protected from DNA damage by the shelterin complex, which includes TRF1, POT1, TRF2, RAP1, TIN2 and TPP1.[9] Removal of individual shelterin subunits can cause the perturbation of T-loop/D-loop structure, resulting in the activation of specific DNA damage response pathways, such as ATM and ATR pathways.[73] TRF1, TRF2 and POT1 directly bind with telomeric DNA, while TIN2, TPP1 and RAP1 perform their protective function through interaction with other shelterin subunits.[73] Our ChIP-qPCR results showed that there was no distinct dissociation of TRF1 from telomeres in NiP-treated cells (Figure S9A), implying that telomeres could be represented by TRF1 foci to investigate the localization of other telomere binding proteins, such as TRF2 and POT1. However, confocal microscopy and ChIP-qPCR results both revealed that the binding of POT1 and TRF2 to the telomeres was significantly reduced after treatment with NiP (Figure S9A-D). The different localization of these telomeric proteins after treatment could be attributed to the difference of binding sites. POT1 binds specifically to 3′ single-stranded G-overhang.[74] TRF2 binds the single-/double-stranded DNA junction and the telomeric duplex DNA, and promotes the 3′-overhang to invade the double-stranded region to form the T-loop structure.[9] In contrast, TRF1 is more prone to bind long tracts of duplex DNA and maintains telomere length.[73] Furthermore, it has been reported that TRF1 possesses higher affinity to telomere DNA than other shelterin subunits, for example, the binding affinity of TRF1 to telomeric DNA is approximately four times higher than TRF2.[75] Therefore, TRF2 and POT1 would be more prone to delocalize from telomeres than TRF1 when telomere DNA forms the structure of G-quadruplex.

It has been confirmed that the anticancer effect of G4 agents is strongly impacted by the length of telomeres, and short telomere is more sensitive to G-quadruplex ligands than longer telomere.[30,57,76] Furthermore, although it is long enough to form T-loop, short telomere is more prone to DNA damage than longer telomere.[73,77] Our results showed that NiP had little effect on normal cells (Figure S16), however, NiP could induce telomere uncapping and telomere DNA damage, leading to 3′-overhang degradation and apoptosis in breast CSCs. These selective effects of NiP on breast CSCs may be due to normal cells possess longer telomeres than cancer cells and CSCs. Thus, targeting telomere G-quadruplex is a promising strategy for eliminating CSCs.

In summary, our results show that one enantiomer of chiral metallohelices, NiP, rather than NiM, has the ability to predominantly reduce cell viability in breast CSCs compared to the bulk cancer cells, and has little effect on normal cells. Further studies demonstrate that NiP is able to induce breast CSC apoptosis at 3 weeks before the total length of telomeres is reduced. The short-term effect of NiP is involved in the loss of telomere associated protein (POT1 and TRF2) from telomeres, which lead to series of telomere DNA damage, degradation of G-overhang and apoptosis. Meanwhile, cell stress caused by NiP induces the translocation of hTERT from nucleus to cytoplasm, accompanied by down-regulation of hTERT and CSC markers (Aldh1, Oct4, Lin28a, Klf4, Nanog and Bmi1), indicative of the reduction of breast CSC traits. Furthermore, NiP, but not NiM, can reduce tumourigenesis of breast CSCs *in vivo*. Our work suggests that chiral recognition of telomere DNA is an efficient way for design of anticancer agents capable of eliminating CSCs.

MATERIALS AND METHODS

Mammosphere culture. For mammosphere culture *in vitro*, MDA-MB-231 and MCF-7 cells were seeded at 5000 cells/ml in 6-well ultralow adherence plates (Corning Inc., USA) in serum-free DMEM/F12 with 20 ng/ml recombinant epidermal growth factor (Sigma, USA), 20 ng/ml basic fibroblast growth factor (Gibco), 4 μg/ml heparin (Sigma), and 1% penicillin/streptomycin (Gibco).[78] Medium was changed every 48 h. After 7 days, mammospheres were subcultured at 5000 cells/ml.

Tumour tissues isolated from PBS, NiM or NiP-treated mice were mechanically dissociated followed by enzymatic dissociation (300 U/ml of collagenase and hyaluronidase for 2 h at 37 °C).[78] After filtration, the cell suspension was centrifuged for 10 min at 5000 rpm. Then the cells were resuspended and plated at 5000 cells/ml in ultralow adherence plates. After 7 days, mammospheres were tested by microscopic examination.

ASSOCIATED CONTENT

Supporting Information

Materials and Methods in detail. Characterization of mammospheres enriched with breast CSCs. TRAP-G4 assay of telomerase activity. Characterization of NiP, NiM, NiP-3, NiM-3, NiP-5 and NiM-5. The effects of NiP-3, NiM-3, NiP-5 and NiM-5 on telomerase activity in breast CSCs. NiP and NiM have no acute toxicity to the mammospheres of MDA-MB-231 and MCF-7 cells. Identification of expression of $TRF2^{\Delta B \Delta M}$. NiP induces DNA damage response in breast CSCs. The effect of NiP on telomere uncapping. NiP induces TdT-Cy3 signal in breast CSCs. NiP induces the emergence of micronuclei in breast CSCs. The phosphorylation level of hTERT induced by NiP. RT-PCR analysis of the expression of CSC makers. The body weights of mice. H&E staining of five organs. The effects of NiP and NiM on HEK293 cells. Primer sequences for PCR experiments.

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