

Halloysite nanotubes/pyrazolo[3,4-*d*]pyrimidine derivatives nanocomposites as potential CDK inhibitors

Marina Massaro,^a Giampaolo Barone,^a Viviana Barra,^a Patrizia Cancemi,^a Aldo Di Leonardo,^a Giancarlo Grossi,^b Fabrizio Lo Celso,^{c,d} Silvia Schenone,^b Cesar Viseras Iborra^{e,f} and Serena Riela^{*,a}

^aDipartimento di Scienze e Tecnologie Biologiche, Chimiche e Farmaceutiche (STEBICEF), University of Palermo, Viale delle Scienze, Ed. 17, 90128 Palermo, Italy. E-mail: serena.riela@unipa.it

^bDepartment of Pharmacy, University of Genoa, Viale Benedetto XV, 16132 Genoa, Italy.

^cDipartimento di Fisica e Chimica (DiFC), University of Palermo, Viale delle Scienze, Ed. 17, 90128 Palermo, Italy.

^dLaboratorio Liquidi Ionici, Istituto Struttura della Materia, Consiglio Nazionale delle Ricerche, (ISM-CNR), Rome, Italy.

^eDepartment of Pharmacy and Pharmaceutical Technology, Faculty of Pharmacy. University of Granada, Campus of Cartuja, 18071 s/n, Granada, Spain.

^fAndalusian Institute of Earth Sciences, CSIC-UGR. Avenida de las Palmeras 4, 18100 Armilla, Granada, Spain.

ABSTRACT. Uncontrolled cell proliferation is a hallmark of cancer as a result of rapid and deregulated progression through the cell cycle. Cyclin dependent kinases (CDKs), when activated by cyclins, are responsible for the transition from one cell cycle phase to the next. Hence, therapeutic strategies that inhibit CDK activity are likely to selectively target tumour cells by blocking their cell cycle.

Pyrazolo[3,4-*d*]pyrimidine derivatives, bioisosteres of drugs like-Roscovitine, could be good candidates as CDK inhibitors. Unfortunately, their low solubility in aqueous media impaired their full development. To overcome these limitations, the use of Halloysite nanotubes (HNTs) as carrier systems should be a smart strategy to deliver these molecules in the body.

In this work we reported a new example of nanocomposites based on HNT/pyrazolo[3,4-*d*]pyrimidine derivatives (Si306 and Si113) as anticancer agents and CDK inhibitors. HNTs/Si306 and HNTs/Si113 nanocomposites were synthesized and characterized. The kinetic release was also investigated. Antitumoral activity was evaluated on three cancer cell lines (HeLa, MDA-MB-231 and HCT116) and the effects on cell cycle arrest in HCT116 cells were evaluated. Finally,

molecular dynamics simulations were performed of the complexes between Si113 or Si306 and the active site of both CDK 1 and 2.

KEYWORDS: halloysite, pyrazolo[3,4-*d*]pyrimidine derivatives, nanocomposites, CDK inhibitors.

INTRODUCTION

Cancer, the second leading causes of death worldwide (about 8.2 million deaths per year, World Health Organization estimates) is a multistep process involving multiple mutations leading to unrestrained cell growth and proliferation as well as genomic and chromosomal instability. Cell proliferation is scheduled by the cell cycle. Cell cycle progression is strictly controlled by checkpoints that are surveillance systems arresting cell proliferation at specific points of the cell cycle if defects occur (DNA damage) which can affect DNA replication (S phase) or chromosome segregation (M phase) (Barnum and O'Connell, 2014). During the cell cycle arrest the cell could repair these defects or, if they are unrepairable, triggers apoptosis.

Cyclin-dependent kinases (CDKs) are a class of serine-threonine kinases associated with each phase of the cell cycle. Among the CDKs, CDK1 regulates mitotic entry in association with Cyclin B (Draetta and Beach, 1988); and CDK2 in complex with Cyclin E, is important for G1-to-S transition (Ohtsubo et al., 1995). Being master regulators of the cell cycle, CDKs are considered promising targets for cancer therapy. Indeed, the use of CDK inhibitors can induce a cell cycle arrest, especially in actively and rapidly proliferating cells like tumor cells, preventing cells from uncontrolled growing (Shapiro, 2017).

A great number of CDK inhibitors has been synthesized and studied as promising antiproliferative agents for cancer therapy (Law et al., 2015). Olomoucine (2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine) (Vesely et al., 1994), and its derivatives, like Roscovitine (Khalil et al., 2015; Poratti and Marzaro, 2019), represent some of the earliest reported specific CDK inhibitors. They have been shown anticancer activity in various *in vitro* and *in vivo* studies (Cicenas et al., 2015). These compounds act as competitive inhibitors for ATP binding (Poratti and Marzaro, 2019). In particular, the key role played by the groups in positions 2, 6 and 9 of the purine heterocycle system has been reported (Figure 1). In detail, the N₆ benzyl substituent makes important interactions outside of the ATP-binding pocket, whereas the N₉ isopropyl and C₂ substituents coincide with the volume occupied by the ribose sugar moiety of ATP (Vesely et al., 1994).

Unfortunately, Roscovitine and some purine like inhibitors are characterized by a low solubility in aqueous media, that implies the use of high dose *in vivo* and as a consequence too high toxicity that impaired their full development (Poratti and Marzaro, 2019).

In the last years, natural and biocompatible nanomaterials able to form supramolecular host/guest interactions with hydrophobic molecules have been identified as an attractive tool for developing nanocomposites able to enhance both the solubility (Lo Meo et al., 2014; Massaro et al., 2016; Rizzo et al., 2017) and the bioavailability of guest molecules in bodily fluids (Lazzara et al., 2017). Halloysite nanotubes (HNTs) are clay minerals, of the kaolin group, characterized by a tubular structure able to host hydrophobic drugs (Yendluri et al., 2017; Massaro et al., 2018a; Gianni et al., 2019; Massaro et al., 2019b). In addition, HNTs are capable to penetrate the cell membrane to deliver drug molecules in the cytosol within 24 h (Vergaro et al., 2010). Several studies reported the use of HNTs as carrier systems for a wide variety of organic molecules (Tully et al., 2016; Massaro et al., 2018b; Massaro et al., 2019a; Homayun and Choi, 2020).

Pyrazolopyrimidines are bioisosteres of purine and consist of a pyrazole five-member ring fused with the pyrimidine moiety, instead of the imidazole moiety in purines (Figure 1). Pyrazolo[3,4-*d*]pyrimidine derivatives, such as Si306 and Si113, have exhibited anticancer activity by competitively binding to the ATP active site of various protein kinases (Chauhan and Kumar, 2013; Cherukupalli et al., 2019). Both Si306 and Si113, indeed, possess substituents at the strategic positions 6, 4, and 1 of the pyrazolo-pyrimidine ring similar to those present in Roscovitine and its derivatives, namely at the positions 2, 6 and 9, of the purine units, respectively (Liebl et al., 2011; Cicen et al., 2015) (Figure 1). Si306 is known as a nanomolar inhibitor selective for the cytoplasmic tyrosine kinase Src, which is overexpressed and/or hyperactivated in a variety of cancers, and is frequently associated with cancer progression (Guarino, 2010). Conversely, Si113 has shown antiproliferative activity on different cancer cells (D'Antona et al., 2015; Talarico et al., 2015; Talarico et al., 2016; D'Antona et al., 2019) both *in vitro* and *in vivo*, but it has not displayed selectivity for a one kind of kinase and thus it could act as a multitargeted inhibitor. Until now, very few studies about the use of pyrazolo[3,4-*d*]pyrimidine derivatives as CDK inhibitors have been reported (Le Brazidec et al., 2012; Cherukupalli et al., 2018; Cherukupalli et al., 2019) in which the drug molecules exerted the suitable CDK inhibition in cells but showed moderate activity in animal models (Le Brazidec et al., 2012), as a consequence of their low solubility in aqueous medium. The use of HNTs as carrier systems for Si306 and Si113 appears to be a smart strategy to transport these molecules into the cell membranes increasing their biochemical and antitumor potency.

In this work we report a new example of nanocomposites based on HNT/pyrazolo[3,4-*d*]pyrimidine derivatives (Si306 and Si113) and we investigated if they could act as CDK inhibitors by studying the cellular cycle arrest. Firstly, we synthesized and characterized the HNTs/Si306 and HNTs/Si113 nanocomposites from a physico-chemical point of view. Then, in order to test the potential use of the halloysite nanocomposites for the delivery of Si306 and Si113, the kinetic release at

physiological conditions was studied. Furthermore, we investigated the *in vitro* antiproliferative activity of the HNTs/Si306 and HNTs/Si113 nanocomposites against human cervical (HeLa), human breast adenocarcinoma (MDA-MB231) and colon (HCT-116) cancer cells lines. The effects of the two nanocomposites on cell cycle phase arrest in HCT-116 cells were also evaluated by flow cytometry. Finally, to confirm the CDK inhibitory effects of Si306 and Si113, we also reported some molecular dynamics simulations of the complexes between pristine pyrazolo[3,4-*d*]pyrimidine derivatives and CDK1 and 2, chosen as CDK protein models (Le Brazidec et al., 2012; Cherukupalli et al., 2018; Cherukupalli et al., 2019). The computational results were in agreement with our experimental observations.

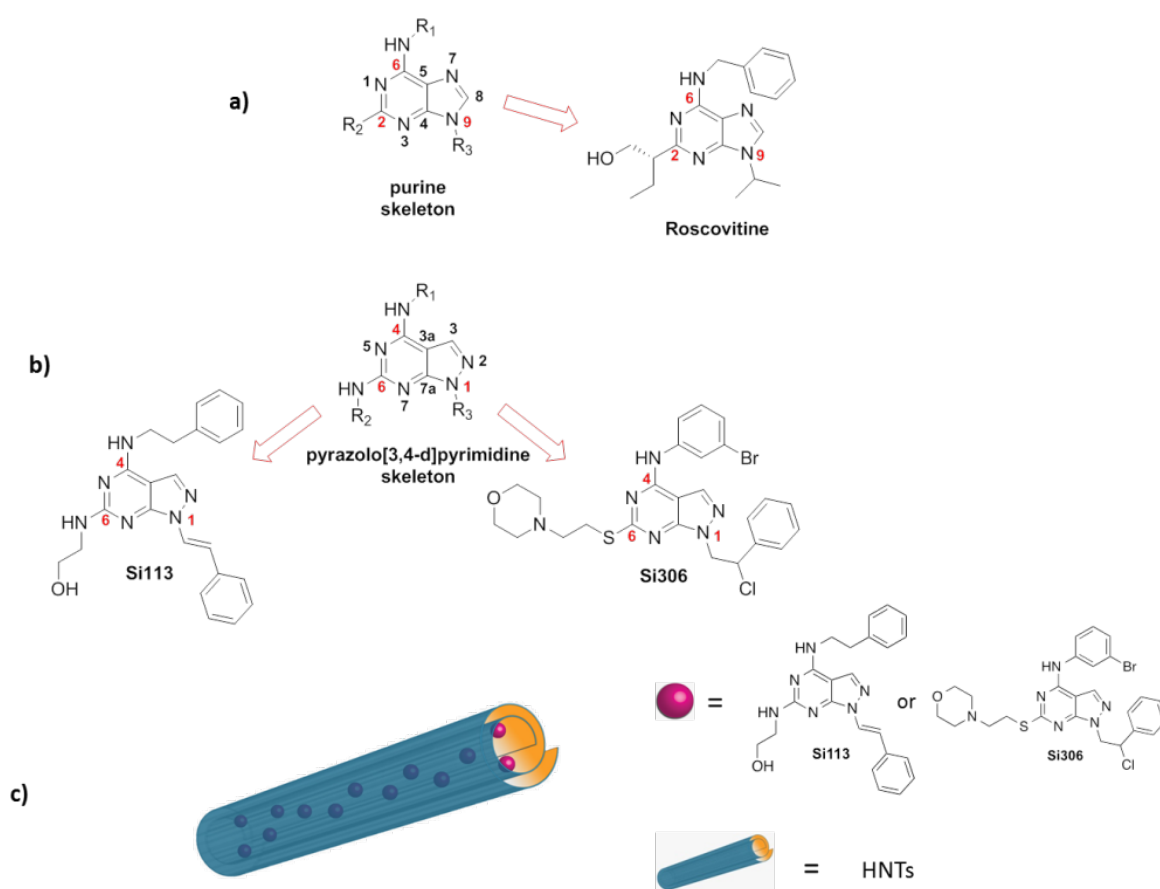


Figure 1. (a) Example of purine based drugs; (b) example of pyrazolo[3,4-*d*]pyrimidine drugs; (c) cartoon representation of HNTs/pyrazolo[3,4-*d*]pyrimidine nanocomposites.

2. Results and discussion

HNTs/pyrazolo[3,4-*d*]pyrimidine derivatives were obtained by the supramolecular loading of Si306 and Si113 compounds, previously synthesized by some of us (Tintori et al., 2015a; Tintori et al., 2015b) (Scheme S.1 and S.2), into HNTs lumen.

In details, the loading of Si306 and Si113 into pristine HNTs was carried out by vacuum cycling of a HNTs suspension in a highly concentrated drug MeOH/DMSO (1:1) solution. Once the vacuum was removed, the solution entered the lumen, and the loaded compound condensed within the tubes. This procedure was repeated several times in order to obtain the highest loading efficiency. After loading, the HNTs/Si306 and HNTs/Si113 nanocomposites were washed several times with water in order to remove free drug molecules. The drug loading of both nanocomposites was estimated by TGA analysis. The loading efficiencies, expressed as the percent amount of drug in the final composite, were ca. 3 wt % (30 mg g^{-1}) for both nanomaterials.

The obtained solid nanocomposites were characterized by FT-IR spectroscopy and thermal properties were evaluated by DSC measurements.

The FT-IR spectra of the HNTs/Si113 and HNTs/Si306 complexes are reported in Figure 2a. As it is possible to note, they both show the typical HNTs vibration stretching bands (Massaro et al., 2019b). Besides these, in the spectrum are present new bands relative to the typical vibration stretching bands of the loaded drugs. In detail, the asymmetric vibration stretching of the aliphatic $-\text{CH}_2$ and $-\text{CH}_3$ groups at ca. 2925 and 2850 cm^{-1} and the bands at around 1580 , 1560 and 1477 cm^{-1} due to the $\text{C}=\text{C}$, $\text{C}=\text{N}$ and $\text{C}-\text{N}$ stretching vibrations of the highly conjugated aromatic systems present in both Si103 (Radi et al., 2011) and Si306 are observable (Tintori et al., 2015a).

In order to evaluate the thermal properties of the HNTs/Si306 and HNTs/Si113 nanomaterials, we performed a DSC analysis. The DSC thermograms of Si306, Si113 and their complex with HNTs are showed in Figure 2b and 2c. The DSC profile of pure Si306 exhibited a sharp endothermic peak due to the melting event (Tintori et al., 2015a), centered at ca. $235 \text{ }^{\circ}\text{C}$ ($\Delta H_f = 100 \text{ J g}^{-1}$). Similarly, Si113 showed an endothermic peak at ca. $107.11 \text{ }^{\circ}\text{C}$ ($\Delta H_f = 100 \text{ J g}^{-1}$) corresponding to the melting point (Radi et al., 2011) of the compound.

As it is reported, pristine HNTs show a wide endothermic phenomenon, in the range $50\text{--}120 \text{ }^{\circ}\text{C}$, ascribed to the dehydration of physisorbed water and interlayer water, followed by another endothermic band ($270\text{--}320 \text{ }^{\circ}\text{C}$) associated with structural decomposition of gibbsite (Aguzzi et al., 2019). The HNTs/Si306 and HNTs/Si113 nanomaterials showed the typical peaks in the thermograms related to the endothermic processes of HNTs and signal is referred to the molecule, confirming the successful loading.

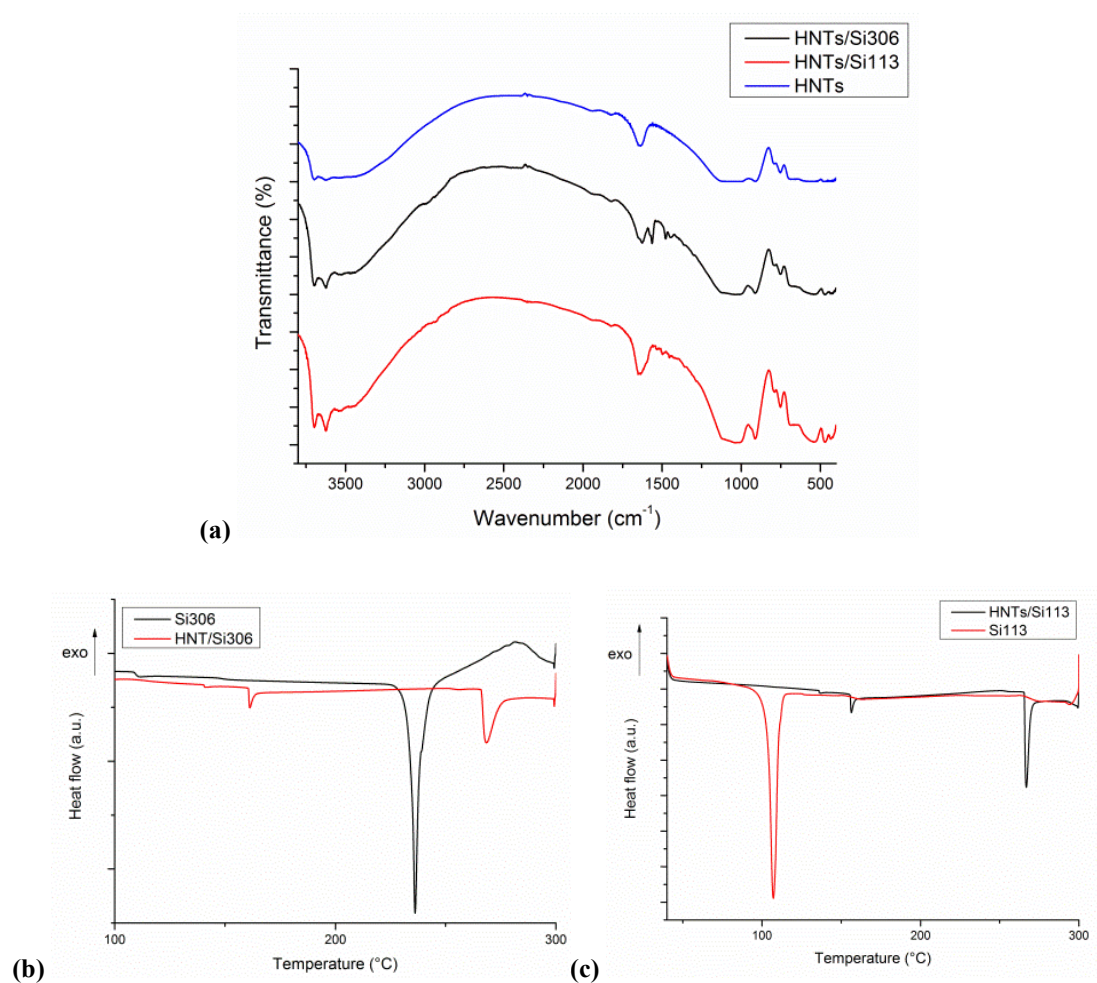


Figure 2. (a) FT-IR spectra of HNTs/Si306 and HNTs/Si113 complexes; (b) DSC profiles of Si306 and HNTs/Si306; (c) DSC profiles of Si113 and HNTs/Si113.

2.1 Kinetic release of Si306 and Si113 from HNTs lumen

To evaluate the performances of the hybrid systems for application in the drug carrier field, the kinetic release of both Si306 and Si113 from HNTs hybrid systems was evaluated by the dialysis bag method using conditions designed to mimic physiological conditions (phosphate buffer pH 7.4, 37 °C) and the obtained kinetic data are shown in Figure 3.

As it is possible to note from Figure 3, only a little amount of Si306 was released from the nanocomposite in ca. 48 h accordingly to its low aqueous solution. Same results were obtained in the case of the release of Si113 even if ca. 15 wt% of the total drug loaded into HNTs lumen is released in 48 h. This latter result, could be due to the slighter enhanced solubility of Si113 compared to Si306 (solubility of 126.5 $\mu\text{g L}^{-1}$ and 3.7 $\mu\text{g L}^{-1}$ for Si113 and Si306, respectively).

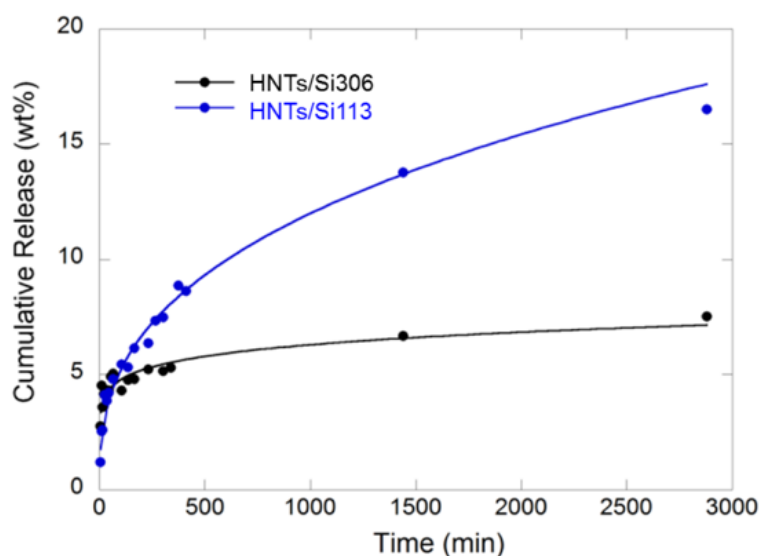


Figure 3. Kinetic release of Si113 and Si306 from HNTs/Si306 and HNTs/Si113 complexes in phosphate buffer pH 7.4 and 37 °C.

The kinetic data obtained were analyzed by first-order and Korsmeyer–Peppas models to deep investigate the release behavior of Si113 and Si306 from such systems. The results showed that data well better fitted, for each case investigated, by a Power fit model indicating that the release is controlled by a Fickian drug diffusion process (n value lower than 0.5 in both cases) (Table 1) in agreement with their release from HNTs lumen.

Table 1. Kinetic parameters for Si113 and Si306 release from HNTs complexes.

	First order model			Power Fit		
	$M_t = M_\infty(1 - e^{-kt})$			$\frac{M_t}{M_\infty} = kt^n$		
	M_∞	k	R^2	k	n	R^2
	(wt%)	(min ⁻¹)		(min ⁻¹)		
HNTs/Si306	3.8 ± 0.4	0.0019±0.0005	0.8922	1.4± 1.0	0.19± 0.07	0.9489
HNTs/Si113	13.8 ± 0.8	0.0015±0.0002	0.9663	1.1± 0.3	0.34± 0.03	0.9884

2.2 Cytotoxic effects on cancer cell lines

The cytotoxicity of the HNTs/pyrazolo[3,4-*d*]pyrimidine derivatives was tested by MTT assay on three different human cancer cell lines namely HCT116, HeLa and MDA-MB-231, derived from colon, cervical and breast cancer, respectively. For comparison, we reported the results obtained in the presence of pristine Si306 and Si113, as well. It should be noted that, these molecules, being highly hydrophobic compounds, were solubilized in DMSO.

The results obtained are shown in Figure 4a-b and the IC₅₀ values, calculated from a dose–response model by fitting sigmoidal curves of the cell percentage inhibition versus the logarithmic concentration of treatments, are reported in Table 2.

As it is possible to note from Figure 4a, both Si306 and Si113 exerted a dose-dependent cytotoxic effect on all tested cell lines; in particular, the colon cancer HCT116 cell line resulted more sensitive to treatments than HeLa and MDA-MB-231, respectively. Furthermore, it was found that Si113 was generally more toxic than Si306, except in the breast cancer cell line MDA-MB-231.

Pristine HNTs showed a good biocompatibility at low dosage in all cell lines investigated. It should be noted that the increased cytotoxicity of HNTs at high dose resembles the findings reported in literature about their safety profile. For instance, HNTs are safe materials in a concentration range up to 500 µg mL⁻¹ for HCT116 cell lines (Ahmed et al., 2015). We observed appreciable HNTs cytotoxicity at concentration of 1200 µg mL⁻¹ (corresponding to a drug concentration in the nanocomposite of 62.5 µM) in agreement with findings reported in previous studies (Vergaro et al., 2010; Ahmed et al., 2015).

As far as the HNTs/Si306 and HNTs/Si113 are concerned, they were tested in a concentration range where HNTs had no cytotoxic effects (ranging from 1 to 62 µM). The obtained results are summarized in Figure 4b. It was found that HNTs/Si113 showed promising cytotoxic activity even at low dosage. Therefore, this result is a good one, since, due to the presence of halloysite, it is possible to disperse the nanocomposite in the culture medium, minimizing some adverse effects of DMSO, as well as the possibility of their use for *in vivo* experiments. HNT/Si306 showed promising cytotoxicity on HCT116 cell lines; conversely, it resulted to be not so toxic on the other cell lines.

In the same experimental conditions the pristine molecules did not show any cytotoxicity probably due to the low solubility in physiological conditions.

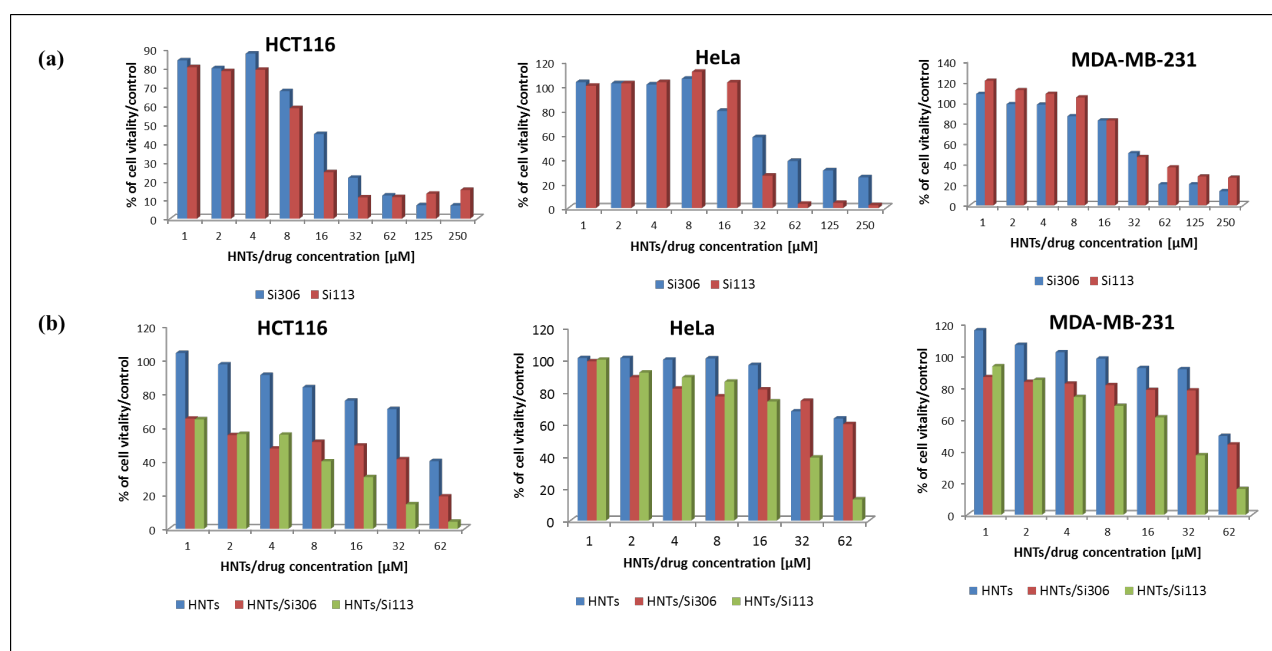


Figure 4. Cytotoxic curves of HCT116, HeLa and MDA-MB-231 cancer cells after 24 h of challenge with increasing concentrations of Si306 and Si113 solubilized in DMSO (a) or loaded in HNTs (HNTs/Si306 and HNTs/Si113) dispersed in aqueous medium (b). For comparison data obtained in the presence of pristine HNTs were also added. The pristine HNTs concentration, expressed as mg/mL of material (ranging from 20 to 1200 $\mu\text{g mL}^{-1}$), is proportional to the HNTs amount in the HNTs/drug derivatives nanocomposites. The cytotoxic curves with relative error bars are reported in SI.

Interestingly, in HCT116 cells (Table 2), the HNTs/Si306 and HNTs/Si113 were more toxic than the free compounds (IC_{50} values of 11.9 ± 1.2 and 6.5 ± 0.7 μM for Si306 and HNTs/Si306 and 8.0 ± 0.6 and 4.7 ± 0.9 μM for Si113 and HNTs/Si113). These results highlight the importance of HNTs as carrier to deliver highly hydrophobic compounds and puts a step forward toward the use of HNTs for biological applications.

Table 2. IC_{50} values (expressed in μM) obtained after 24 h of treatments for HCT116, HeLa and MDA-MB-231 cancer cell lines.

	$\text{IC}_{50} \mu\text{M} \pm \text{SD}$		
	HCT116	HeLa	MDA-MB-231
Si306 ^a	11.9 ± 1.2	47.2 ± 2.1	29.8 ± 1.4
Si113 ^a	8.0 ± 0.6	25.7 ± 1.4	42.8 ± 1.5
HNTs/Si306	6.5 ± 0.7	not reached	not reached
HNTs/Si113	4.7 ± 0.9	23.6 ± 1.3	15.7 ± 1.0

^a Compounds solubilized in DMSO.

2.3 Cell Cycle Analysis

Since few studies about the use of pyrazolo[3,4-*d*]pyrimidine derivatives report their use as CDK inhibitors, first of all we performed a preliminary screening of cell cycle in the presence of pristine molecules, by a cytofluorimetry analysis with propidium iodide staining (PI).

On the basis of the cytotoxic results, we performed the aforementioned analysis on the colorectal cancer cell line HCT116 since it was the most sensitive to treatments. Growing cells were treated for 24 hours with increasing concentrations of Si306 and Si113. Following PI staining the cytofluorimetric profiles showed that increasing concentrations of Si113 lead to a progressive accumulation of cells in the G1 phase of the cell cycle (Figure 5a). On the other hand, only treatment with high doses of Si306 (10 μ M) induced an accumulation of cells in G1.

This is in accord with MTT results that showed a lower cytotoxicity for HCT116 cells treated with Si306 compared to the treatment with Si113. The G1-arrest with both treatments is compatible with inhibition of CDK2 which is involved in the G1-S progression. The difference between the efficiency of the two compounds could be due to the different binding of Si113 and Si306 with CDK2, see *infra*. These results are in agreement with the CDK2 inhibition activities of purine and pyrazolo[3,4-*d*]pyrimidine derivatives (Liebl et al., 2011; Cherukupalli et al., 2018; Cherukupalli et al., 2019).

Pristine HNTs did not show any effect on the cell cycle progression, in all range of concentration investigated.

The cytofluorimetric profiles showed that already at 24 hours treatment with HNTs/Si113 has a similar effect on HCT116 cells as the pristine molecule (Figure 5b). Instead, HNTs/Si306 treatment did not induce a G1 arrest even at 10 μ M. This is most likely related to the observed slower release of the compound from HNTs lumen.

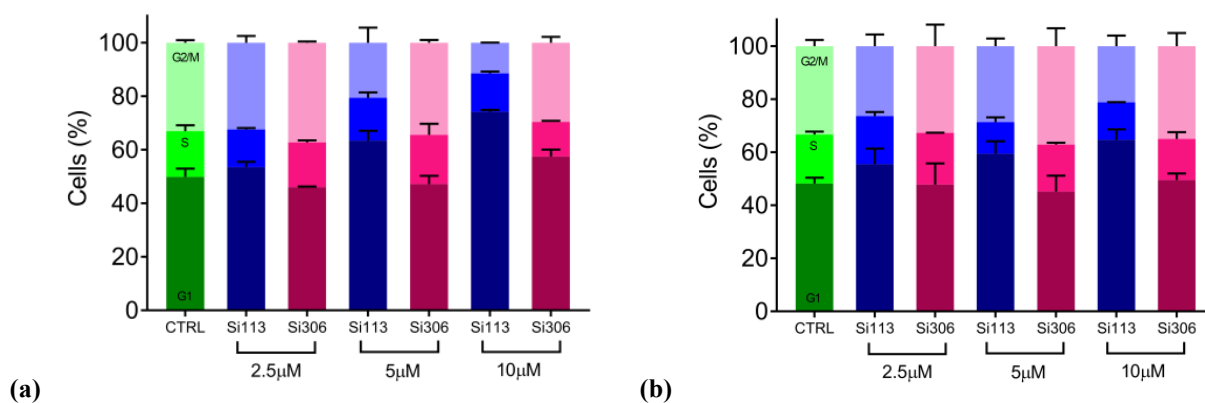


Figure 5. Percentages of HCT116 cells in each phase of the cell cycle (G1, S, G2/M) after treatment with (a) pristine Si113 and Si306; (b) Si113 and Si306 in HNTs/Si113 and HNTs/Si306 nanocomposites at 24 h. The graph represents the mean and SEM of two replicates.

2.4 Molecular modeling

To support the experimental data above discussed, we performed molecular dynamics (MD) simulations of the binding between Si306 and Si113 with the CDK (1 or 2) catalytic domain.

As recently reported, CDK1 and CDK2 protein active sites have about 89% similar residues and 74% identical residues (Gallorini et al., 2012). The results of the MD simulations show that Si113 binds with CDK2 in only one possible orientation (Figure 6), since two different starting positions of the ligand did not result in a stable binding. For Si306, instead, two over three attempts provided stable equilibrium structures (Figure 7a-b and d). This finding supports the hypothesis that a more selective binding occurs between Si113 and CDK2, compared to Si306. Moreover, the analysis of the superimposed structures of the complexes of Si113 and Si306 with CDK2 (Figure 7c) shows that Si113 is more deeply inserted in the binding site compared to Si306. The different binding ability of the two ligands is attributable essentially to the different affinity toward residues present in the binding site of both proteins. In details, Si113 makes a H-bond with Asp86 and two hydrophobic interactions with Lys20 and Phe82 (Figure 6, inset). In the other hand, Si306 makes only one H-bond with Glu12 in one orientation and two H-bonds with Gln131 and Thr158 (Figure 7b). Analogous structures for of the complexes with CDK1 and their comparison with the binding with CDK2 are reported in the Supporting Information (Figures S1, S2 and S3). The superimposed structures of the two proteins, CDK1 and CDK2, complexed with Si113 and with Si306 (Figure S2) show that the binding site is the same in both proteins. However, also in this case it is possible to conclude that the binding of both Si113 and Si306 with CDK1 is not selective, since the three starting poses resulted in three different equilibrium structures. The difference is to be attributable to the different aminoacidic residues present in the two binding sites. Therefore, the computational results are in agreement with our experimental observations. Finally, interestingly, the binding site of Si113 with CDK2 is exactly the same of that found for Roscovitine through X-ray crystallography (pdb ID: 2a4l) (De Azevedo et al., 1997; Cicenas et al., 2015). This result nicely supports the occurrence of a similar mechanism for CDK2 inhibition.

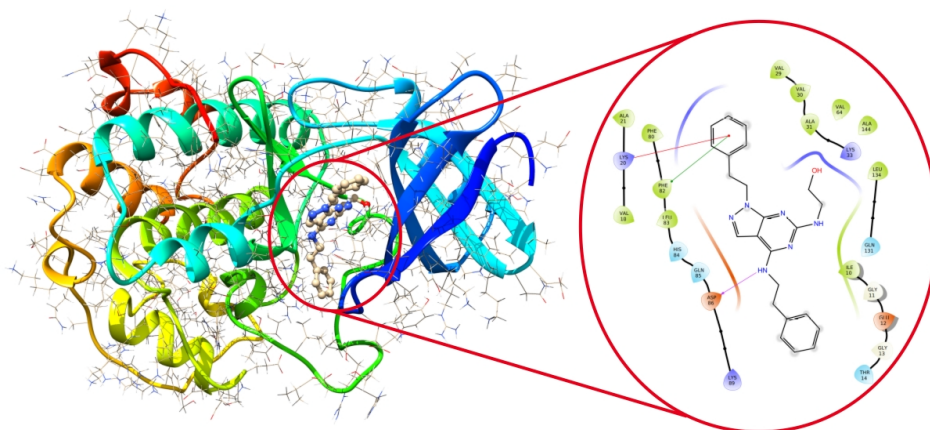


Figure 6. Most recurrent structure of the complex between Si113 and CDK2, obtained at the equilibrium in 200 ns of MD simulation. In the inset: 2D structure showing H-bond and hydrophobic interactions with the indicated aminoacid residues.

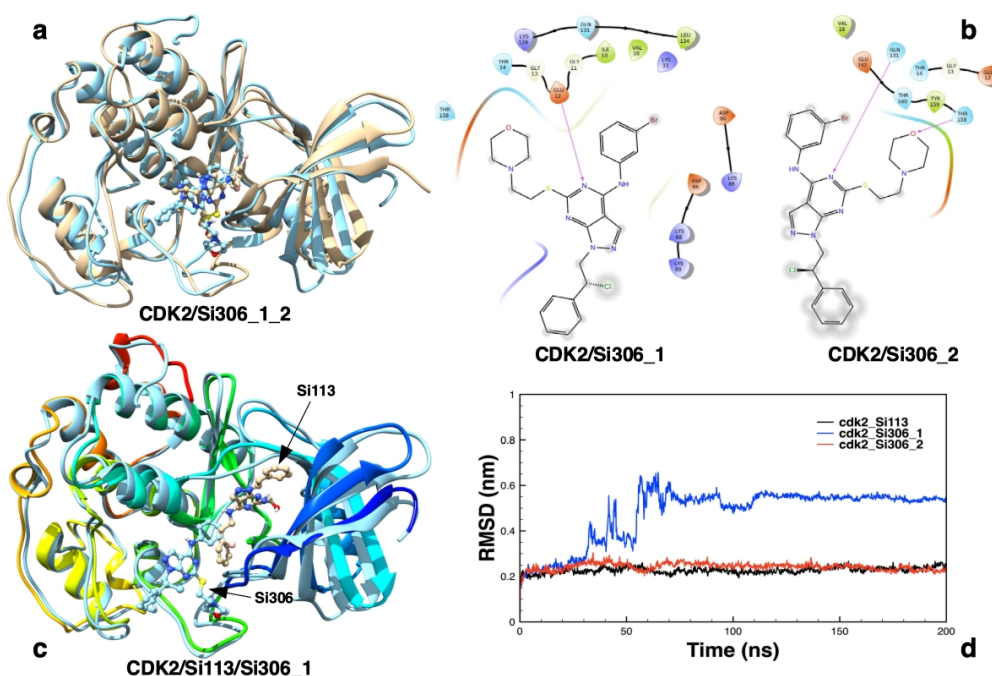


Figure 7. a: Superimposed structures of the two complexes between Si306 and CDK2, obtained at the equilibrium in 200 ns of MD simulation. b: 2D structures showing H-bond and hydrophobic interactions with the indicated aminoacid residues. c: Superimposed structures of the two complexes between Si113 and Si306 with CDK2. d: RMSD plot.

3. Experimental

Halloysite was purchased from Merck and used without further purification. Halloysite has an average tube diameter of 50 nm and inner lumen diameter of 15 nm. Typical specific surface area is $65 \text{ m}^2 \text{ g}^{-1}$; pore volume of $\sim 1.25 \text{ cm}^3 \text{ g}^{-1}$; refractive index 1.54 and specific gravity 2.53 g cm^{-3} .

Thermogravimetric analyses (TGA) were performed with a Mettler Toledo, equipped with sensor and FRS5 microbalance (precision 0.1 μg) and FP89 software package, using a heating rate of 5 $^{\circ}\text{C}/\text{min}$ in the 30–800 $^{\circ}\text{C}$ temperature range.

FT-IR spectra (KBr) were recorded with an Agilent Technologies Cary 630 FT-IR spectrometer. Specimens for these measurements were prepared by mixing 5 mg of the sample powder with 100 mg of KBr.

DSC measurements were carried out on a DSC Q200 calorimeter interfaced to a TA Thermal Analyst 3100 controller connected to an RCS90 cooling system. Heating cycles were done in a 50 mL/min stream of nitrogen. Samples were weighed in T-zero aluminum pans. For each sample, after equilibration of the sample at 25 $^{\circ}\text{C}$, DSC measurements were performed using a temperature ramp with a rate of 10 $^{\circ}\text{C}/\text{min}$ from 30 $^{\circ}\text{C}$ to 300 $^{\circ}\text{C}$.

Si113 and Si306 were synthesized as reported elsewhere (Tintori et al., 2015a; Tintori et al., 2015b).

3.1 Computational details

The coordinates of the protein models were taken from RCSB PDB data bank (www.rcsb.org; pdb codes 4y72 and 2c6m, for CDK1 and CDK2 respectively). Three starting poses for each of the complexes of Si306 and Si113 with both CDK proteins, for a total of 12 systems, were obtained through molecular docking calculations, using the Autodock Vina package (Trott and Olson, 2010). MD simulations were performed using the GROMACS 2019.4 package (Van Der Spoel et al., 2005; Hess et al., 2008). Bonded and non-bonded interactions for the CDK proteins were described using an all-atoms Amber99SB-ILDN force field (Lindorff-Larsen et al., 2010). The force field for the CDK inhibitors were obtained from the Merck Molecular ForceField (MMFF), with van der Waals parameters taken from the closest atom type in CHARMM22, through the SwissParam web interface (Zoete et al., 2011). The simulations for the various systems were performed using a cubic box of explicit TIP3P water, with a distance between the walls and CDK atoms of 10 Å. Periodic boundary conditions were applied. Force field parameter files and initial configuration for the systems were created by GROMACS utilities programs. The equilibration procedure was done in several steps, starting from a 2 ns NVT simulation at 300K with the system heavy atom positions restrained to equilibrate the solvent around it, followed by a NPT run at 300 K and pressure at 1 bar, for a 2 ns run. After the equilibration phase, there was a NPT production run (total length can be derived from RMSD plot, depending on the system); the trajectory was saved at a frequency of 10 ps to evaluate dynamical and structural properties. The simulations were always checked versus the root mean square displacement (RMSD) and the energy profile. The g-cluster tool, within the GROMACS package, was used to determine the most frequent structure in the equilibrium phase of the MD simulation. During the production run for the temperature coupling, we used a velocity

rescaling thermostat (Bussi et al., 2007) (with a time coupling constant of 0.1 ps), while for the pressure coupling, we used a Parrinello–Rahman barostat (Parrinello and Rahman, 1981) (1 ps for the relaxation constant). The Leap-Frog algorithm with a 2 fs time step was used for integrating the equations of motion. Cut-offs for the Lennard-Jones and real space part of the Coulombic interactions were set to 10 Å. For the electrostatic interactions, the Particle Mesh Ewald (PME) summation method (Darden et al., 1993; Essmann et al., 1995) was used, with an interpolation order of 4 and 0.16 nm of FFT grid spacing. Selected images and geometry manipulation were done using Chimera (Pettersen et al., 2004), PyMOL (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC) and Maestro (Schrödinger Release 2020-2: Maestro, Schrödinger, LLC, New York, NY, 2020).

3.2 Synthesis of the HNTs/Si113 and HNTs/Si306 complexes

To a dispersion of HNTs (100 mg) in water (5 mL), Si113 or Si306 (1 mL of 1×10^{-2} M MeOH/DMSO (1:1) solution) was added. The suspension was sonicated for 5 min, at an ultrasound power of 200 W and at 25 °C and was left under stirring for 18 h at room temperature. After this time, the solvent was filtered off, under vacuum, and the powder was washed several times with water and then dried.

3.3 Kinetic Release

The release of the Si306 or Si113 from the HNTs was done as follows: 20 mg of the sample were dispersed in 1 mL of dissolution medium and transferred into a dialysis membrane (Medicell International Ltd MWCO 12-14000 with a diameter of 21.5 mm). Phosphate buffer (0.05 M, pH 7.4) was used as the release medium. Subsequently the membrane was put in a round bottom flask containing 9 mL of the release medium at 37 °C and stirred. At fixed time, 1 mL of the release medium has been withdrawn and analyzed by UV-*vis* measurements. To keep constant the volume of the release medium, 1 mL of fresh solution has been used to replace the collected one. Total amounts of drug released (F_t) were calculated as follows:

$$F_t = V_m C_t + \sum_{i=0}^{t-1} V_a C_i \quad (\text{Eq. 1})$$

where V_m and C_t are the volume and the concentration of the drug at time t . V_a is the volume of the sample withdrawn and C_i is the drug concentration at time i ($i < t$).

3.4 Cell cultures

Colon cancer HCT116 (kindly provided by Dr. B. Vogelstein, John Hopkins University, Baltimore, MD), cervix adenocarcinoma HeLa and breast cancer MDA-MB-231 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U mL⁻¹ penicillin and 100 mg mL⁻¹ streptomycin. Cells were maintained at 37° C in a

humidified atmosphere with 5% CO₂, as previously reported.(Pucci-Minafra et al., 2008; Veneziano et al., 2016; Cancemi et al., 2019)

3.5 Cell Viability Assay

Cell viability was assessed by MTT assay, as previously described (Cancemi et al., 2017; Raimondi et al., 2020). Briefly, the cells (plated at 5×10^3 cells/well in 96-well plates) were incubated for 24 h prior to treatment for further 24 h of different concentrations of Si113, Si306, HNTs, HNTs/Si113 and HNTs/Si306. After the treatment cells were washed with PBS and 20 μ L of Thiazolyl Blue Tetrazolium Bromide (5 mg mL^{-1}) in PBS was added to each well and further cultured for 2h at 37 °C in CO₂ incubator. After removing the medium containing MTT and three washes with PBS, 100 μ L of warm DMSO was added to each well to dissolve formazan. The absorbance was recorded at 570 nm using a microplate reader (Spark® 20M Tecan Trading AG, Switzerland). Percentage of cell viability with respect to untreated control cells (vehicle control) was calculated after subtraction of the blank. The IC₅₀ values, that is the concentration necessary to inhibit the 50% of cell growth, was calculated using a dose-response model, obtained from sigmoidal fitting of response curves of percent inhibition versus logarithmic concentration of each compound, using Graph Pad Prism software as already described (Billeci et al., 2020; Saladino et al., 2020). Each result represents the mean value of three different experiments performed in triplicate.

3.6 Cell cycle analysis

Growing HCT116 cells were treated with 2.5, 5, 7, 10 μ M of the free compounds (Si113 and Si306), or loaded onto halloysite (HNTs/Si113 and HNTs/Si306) for 24 hours. DMSO was used as control for the free compounds, while HNTs at the same concentrations were used as control for the compounds loaded onto halloysite. At the end of the treatment the cells were collected in PBS and fixed in cold 70% ethanol. Fixed cells were then washed in cold PBS, stained with a solution of 4 μ g/mL Propidium Iodide and 40 μ g/mL RNase A in PBS as previously performed (Costa et al., 2016). and acquired with a FACSCanto and FACSDiva Software (Becton Dickinson). Experiments were repeated twice, and 20000 events were analyzed by FlowJo software. The discrimination of cell doublets from singlets was made by plotting the DNA dye (PI) channel Width versus Area in a dot plot graph as described previously (Fragkos et al., 2019). The gating was done by distinguishing 2n and 4n content cells (G1 and G2/M phase respectively) on the base of DNA content.

4. Conclusions

In summary, we put a step forward to the use of pyrazolo[3,4-*d*]pyrimidine derivatives as drugs by an innovative smart strategy based on nanocontainers which allows to increase their bioavailability. A biocompatible natural clay mineral, namely halloysite, was chosen as nanocontainer and it was used to develop HNTs/pyrazolo[3,4-*d*]pyrimidine derivatives nanocomposites. They were

characterized by a physico-chemical point of view, the kinetic release of molecules and the antiproliferative activity towards different cell lines were evaluated. The release profiles were in agreement with the different molecule solubility and the antiproliferative studies showed promising cytotoxic activity towards HCT116 cell lines. Furthermore, the cell cycle was also investigated, showing cells arrested in the G1 phase likely caused by CDK2 inhibition. Finally, molecular dynamics studies support the experimental results and confirm in particular the selective interaction of the pristine Si113 with the active site of CDK2.

Altogether our findings contribute to increase the knowledge of the mode of action of these promising molecules as CDK2 inhibitors and provide a basis for the use of HNTs as carrier systems for Si306 and Si113 delivery.

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