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Abstract

Granulatimide and isogranulatimide, natural products isolated from an ascidian, were found to be abrogators of the cell cycle G2-M phase checkpoint by inhibition of Checkpoint 1 kinase (Chk1). In the course of structure–activity relationship studies on granulatimide analogues, we have synthesized a series of bis-imides, in which the imidazole moiety was replaced by an imide heterocycle. Various modifications have been introduced on one or both imide heterocycles, on the benzene ring, and on the indole nitrogen. Moreover, aza bis-imide analogues were synthesized in which the indole moiety was replaced by a 7-azaindole. Compared to those of granulatimide and isogranulatimide, the Chk1 inhibitory activities of some of the bis-imide carbazoles were stronger. In particular, 1,3,4,6-tetrahydro-10-hydroxy-7*H*-dipyrrolo[3,4-*a*:3,4-*c*] carbazole-1,3,4,6-tetraone **11** exhibited an IC_{50} value on purified full length Chk1 of 2 nM, which makes it a more potent Chk1 inhibitor than granulatimide and isogranulatimide. To get an insight into the selectivity of this new family of compounds, the inhibitory activities of 1,3,4,6-tetrahydro-7*H*-dipyrrolo[3,4-*a*:3,4-*c*]carbazole-1,3,4,6-tetraone **A** have been evaluated on a panel of 15 kinases, the strongest inhibitory potency was found for Chk1. The inhibitory activities of compounds **A**, **5** and **11** toward Src tyrosine kinase and the cytotoxicity of various tumor cell lines were also evaluated.

Keywords: Granulatimide; Isogranulatimide; Antitumor agent; Chk1 inhibitor

1. Introduction

Cell division of eukaryotic cells is a highly regulated process in which many pathways participate to maintain genome integrity. When DNA is damaged, cells respond by activating checkpoint pathways that cause an arrest in the cell cycle progression to allow time for DNA repair. Ataxia-Telangiectasia Mutated (ATM) and Ataxia-Telangiectasia Related (ATR) kinases are central components of the DNA damage response in the S phase and G2-M checkpoints, which activate Chk1 and Chk2 kinases (Zhou and Elledge, 2000; Luo et al., 2001; Molinari, 2000). Chk1 kinase, initially identified in 1993 (Walworth et al., 1993) has triggered considerable interest in the past decade as a target for the design of novel antitumor agents. Indeed, it has been shown that G2 abrogation can sensitize *p53*-mutated cells to DNA-damaging cancer therapeutic agents. In more than 50% of human tumors, p53 is mutated, therefore leading to G1 checkpoint deficiencies. It is believed that subsequent inhibition of the G2 checkpoint in combination with a DNA-damaging agent could selectively induce the death of cancer cells (Suganuma et al., 1999; Prudhomme, 2004).

Various Chk1 inhibitors have been reported in the literature (Anderson et al., 2003) and more recently in several patents (Prudhomme, 2006; Tao and Lin, 2006). Among these molecules aminopyrazoles, pyrimidines and ureas possess IC_{50} values toward Chk1 in the nanomolar range.

The crystal structure of the human Chk1KD apoenzyme and its binary complex with an ATP analogue has been determined (Chen et al., 2000). More recently, the crystal structures of Chk1 in complex with several ATP competitive inhibitors, staurosporine, UCN-01, SB-218078, and isogranulatimide have also been resolved (Zhao et al., 2002; Jiang et al., 2004) (Fig. 1).

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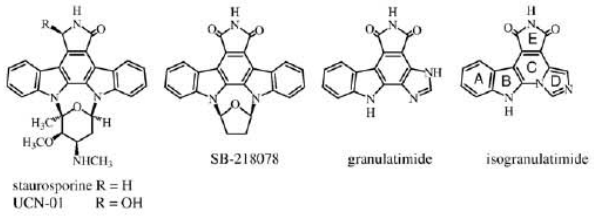

Fig. 1. Chemical structures of staurosporine, UCN-01, SB-218078, granulatimide and isogranulatimide.

Granulatimide and isogranulatimide are natural compounds isolated from the ascidian *Didemnum granulatum* possessing Chk1 inhibitory properties (Berlinck et al., 1998; Roberge et al., 1998; Jiang et al., 2004). In the crystal structure of Chk1 in complex with isogranulatimide, as well as in those of Chk1 in complex with staurosporine, UCN-01, SB-218078, hydrogen bonds are observed between the lactam or the imide NH of the drug and the carbonyl of Glu^{85} and between the carbonyl of the imide or lactam and Cys^{87} in the ATP binding site of the enzyme. Isogranulatimide isomers and closely related compounds have been synthesized (Piers et al., 2000; Yoshida et al., 2002, 2003).

In the course of structure–activity relationship studies on granulatimide analogues, we have synthesized compounds in which the imidazole heterocycle was replaced by a pyrrole or an imide moiety, as well as compounds in which the indole unit was replaced by a 7-azaindole unit (Hugon et al., 2003a,b,c). Concerning the bis-imide analogues, substitutions were carried out either on the indole moiety or on the imide nitrogens (Fig. 2). The syntheses were performed either by conventional methods or using parallel solution-phase method and microwave irradiation (Hénon et al., 2005, 2006a,b,c). The Chk1 inhibitory activities of several bis-imides substituted in the 10 position of the indole moiety with various saturated and unsaturated side chains, synthesized via palladium catalysed cross-coupling reactions, have been evaluated (Hénon et al., 2006c). The most efficient Chk1 inhibitor was compound **B** (Fig. 2), which has a methyl substituent at the 10 position with an IC_{50} value toward Chk1 of 8 nM.

In this paper, the Chk1 inhibitory activities of bis-imide granulatimide analogues modified on the indole moiety, on the imide heterocycles, as well as those of 7-azaindole bis-imides are described (Fig. 3, Table 1) together with their antiproliferative activities toward five tumor cell lines in vitro: murine leukemia L1210, human DU145 prostate carcinoma, A549 non-small cell lung carcinoma and HCT116 and HT29 colon carcinomas (IC_{50} μM). The possible interactions with the ATP binding site of Chk1 were investigated by molecular modelling for some of our bis-imides. To get insight into the kinase selectivity of this family of compounds, the inhibitory activities of the nude bis-imide **A** were evaluated with a panel of 15 kinases. The percentages of activity of various kinases: Checkpoint 1 kinase (Chk1), AMP-activated protein kinase (AMPK), Ca^{2+}/calmodulin dependent protein kinase II (CAMKII), casein kinase I (CKI), fibroblast growth factor receptor 3 (FGFR3), glycogen synthase kinase 3 (GSK3), lymphocyte-specific protein tyrosine kinase (LCK), MAP kinase 1 (MAPK1), mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2), kinase responsible for site specific phosphorylation of BAD (P70S6K), protein kinase A (PKA), protein kinases C β, α, ε isoforms (PKCβ, PKCα, PKCε) were determined at a concentration of 1 μM (Table 2). The inhibitory activities of the bis-imides **A**, **5**, and **11**, toward Src tyrosine kinase were also examined. The percentages of Src kinase inhibition were determined (Table 3).

2. Materials and methods

2.1. Kinase inhibition assays

Chk1 inhibition assays: Human Chk1 full-length enzyme with a N-terminal GST sequence was either purchased from

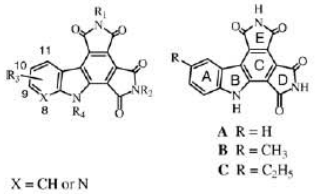

Fig. 2. Bis-imide granulatimide analogues.

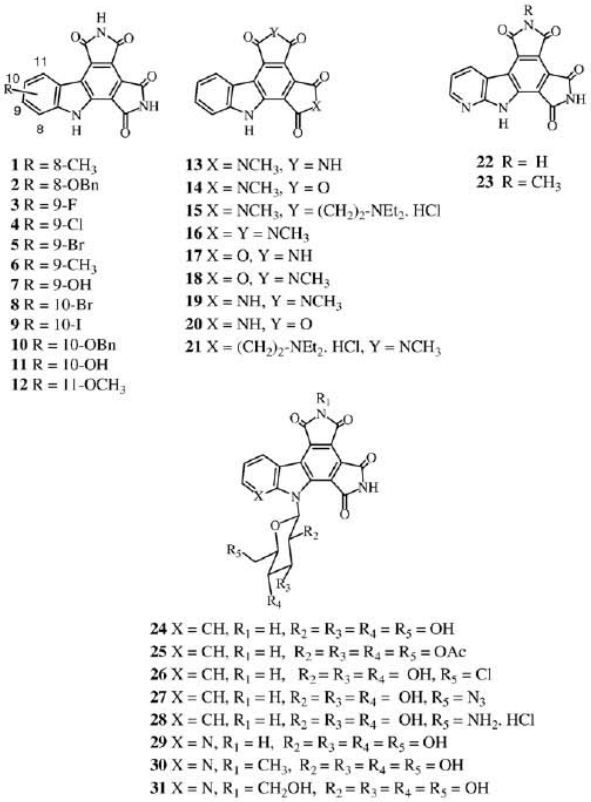

Fig. 3. Structure of the various bis-imides.

Table 1
Percentages of Chk1 inhibition at a drug concentration of 10^{-5} M, IC_{50} values (μM) toward Chk1

Compound	% Inhibition	IC_{50}	L1210[a]	DU145[a]	A549[a]	HCT116[a]	HT29[a]
	Chk1 at 10^{-5} M	Chk1 (μM)					
Granulatimide	93.9	0.081	2.8	2.8	11.4	nd	5.7
Isogranulatimide	89.7	0.438	10	13.1	18.1	nd	13.7
A	94.4	0.017	32.7	53.6	65.7	nd	9.7
B	91.1	0.0087	1.8	nd	nd	2.2	3.29
C	75.8	5	5.9	nd	nd	7.6	16.4
1	44.2		18.5	nd	nd	40.9	84.3
2	10.9		2.7	17.1	nd	nd	18.6
3	87.0	0.068	7.3	nd	nd	8.6	20.0
4	91.1	0.0345	0.9	nd	nd	1.5	9.8
5	80.6	0.011	0.7	3.06	nd	nd	3.1
6	74.9	0.0366	2.2	nd	nd	4.9	>100
7	94.4	0.135	42.2	nd	nd	16.4	42.5
8	90.2	0.0327	3.61	10.1	nd	nd	20
9	nd		nd	nd	nd	nd	nd
10	53.9	5	3.37	3.7	nd	nd	3.7
11	97.1	0.002	54.7	71.9	62.9	nd	nd
12	23.1		17.1	nd	nd	14.5	26.6
13	75.7	5	5.47	6.77	6.47	nd	10.6
14	39.6		26	20.7	23.8	nd	11.2
15	11.89		45.3	43.1	31.7	nd	46.7
16	22.2		8.3	16	9.5	nd	12.9
17	46.7		>50	>50	>50	nd	24.2
18	24.3		67.5	>100	>100	nd	56.1
19	30.7		61.2	23.7	20.1	nd	30.3
20	−6.6		11.9	nd	nd	10.7	33.0
21	13.9		59.3	67.8	44.1	nd	51.5
22	67.6	5	17.9	nd	nd	7.0	10.6
23	−17.5		21.7	12.2	nd	nd	9
24	83.2	0.145	45.4	nd	nd	68.5	56.1
25	36.8		64.3	nd	nd	89.0	>100
26	76.5	0.387	40.8	nd	nd	54.2	42.9
27	74.4	2.15	63.8	nd	nd	94.5	>100
28	56.8	1.44	83.9	nd	nd	nd	46.3
29	14.4		109	nd	nd	73.6	21.8
30	14.9		18.1	nd	nd	29.3	29.3
31	56.0	5	17.9	nd	nd	7.0	10.6

In vitro antiproliferative activities against five tumor cell lines: one murine leukemia L1210, and human DU145 prostate carcinoma, A549 non-small cell lung carcinoma and HCT116 and HT29 colon carcinoma (IC_{50} μM).
nd: not determined.
[a] The cytotoxicities could be due to breakdown products.

Upstate Biochemicals (No. 14-346). Assays for compound testing were based upon the method described by Davies et al. (2000).

Src inhibition assays: Inhibitors were diluted with a Tecan Evo150 robot. The kinase assay was performed with 4 μl of inhibitor (10% dimethylsulfoxide (DMSO)), 10 μl of kinase assay buffer 4× concentrated (80 mM $MgCl_2$, 200 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.4 mM ethylenediamine-tetraacetic acid (EDTA), 2 mM DL-dithiothreitol (DTT)), 10 μl substrate peptide (KVEKIGEGYYGVVYK, 370 nM) and 6 μl Src kinase (stock GTP purified diluted with 1× kinase assay buffer to 200 nM). 10 μl co-substrate (40 μM ATP with 0.2 μCi P^{33}-γ-ATP) was added with a Precision 2000 (Biotek Robotic). The assay was incubated for 20 min at 30 °C then stopped by adding 200 μl 0.85% orthophosphoric acid, then transferred to a phosphocellulose

Table 2
Inhibitory activity of the lead bis-imide **A** toward 15 kinases

Compound	Chk1	AMPK	CAMKII	CDK2/cyclin A	CKI	FGFR3	GSK3	LCK	MAPK1	MAPKAPK2	P70S6K	PKA	PKCβ	PKCα	PKCε
A	5	48	84	42	97	35	11	83	86	95	81	99	91	67	91

Percentages of activity of kinases at a drug concentration of 1 μM: Checkpoint 1 kinase (Chk1), AMP-activated protein kinase (AMPK), Ca^{2+}/calmodulin dependent protein kinase II (CAMKII), casein kinase I (CKI), fibroblast growth factor receptor 3 (FGFR3), glycogen synthase kinase 3 (GSK3), lymphocyte-specific protein tyrosine kinase (LCK), MAP kinase 1 (MAPK1), mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2), kinase responsible for site specific phosphorylation of BAD (P70S6K), protein kinase A (PKA), protein kinases C β, α, ε isoforms (PKCβ, PKCα, PKCε).

filter microplate (Whatman-P81). The plate was washed 3 times with 200 µl 0.85% orthophosphoric acid dried with 200 µl acetone. The remaining activity was measured on a Topcount with 25 µl scintillation solution (Packard UltimaGold).

Inhibition assays toward other kinases were performed by Upstate's kinase profiler screening service (Dundee, Scotland).

2.2. Growth inhibition assays

Tumor cells were provided by American Type Culture Collection (Frederick, MD, USA). They were cultivated in RPMI 1640 medium (Life Science technologies, Cergy-Pontoise, France) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10 mM HEPES buffer (pH=7.4). Cytotoxicity was measured by the microculture tetrazolium assay as described (Léonce et al., 1996). Cells were continuously exposed to graded concentrations of the compounds for four doubling times, then 15 µl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added to each well and the plates were incubated for 4h at 37 °C. The medium was then aspirated and the formazan solubilized by 100 µl of DMSO. Results are expressed as IC_{50}, concentration which reduced by 50% the optical density of treated cells with respect to untreated controls.

2.3. Molecular modelling

All molecular mechanics calculations were performed by the Macromodel [26] molecular modelling software. We used as model the complex structure of CHK1/STAUROSPORINE [6] downloaded from the Protein Data Bank (1NVR file).

Energy minimisation was done with AMBER force field [27, 28] using the Truncated Newton Conjugate Gradient method (TNCG).

3. Results

3.1. Inhibitory activities toward Chk1

The percentages of Chk1 inhibition were evaluated at a drug concentration of 10^{-5} M. For the most efficient compounds, the IC_{50} values were determined and compared with those of granulatimide, isogranulatimide and compounds **A**–**C** (Hénon et al., 2006c), bearing a methyl or an ethyl group at the 10 position, respectively (Table 1). The cytotoxicities toward five tumor cell lines: one murine leukemia L1210 and four human tumor cell lines, DU145 prostate carcinoma, A549 non-small cell lung carcinoma and HCT116 and HT29 colon carcinoma. The IC_{50} values are expressed in µM.

Concerning the substitutions at various positions of the indole moiety (compounds **1**–**12**), all the compounds tested exhibit strong inhibitory activities toward Chk1, except compounds **1** and **2**, substituted in the 8 position. Since a methyl group in the 9 or 10 positions is compatible with Chk1 inhibition (compounds **6** and **B**), it can be assumed that substitutions in the 8 position are not favoured.

Table 3
Inhibitory activities of bis-imides **A**, **5** and **11** toward Src kinase

Compounds at a drug concentration (µM)	**A** (10 µM)	**5** (4 µM)	**11** (4 µM)
% Inhibition	35.6	66	32.3

Percentages of Src inhibition at a given drug concentration.

Substitutions in the 9 position with halogens, methyl or hydroxy substituents are compatible with Chk1 inhibition but, compared with the unsubstituted compound **A**, they did not induce significant improvement. With halogen atoms in the 9 position, the following sequence of efficiency is observed Br (compound **5**)>Cl (compound **4**)>F (compound **3**).

Substitutions in the 10 position are favoured (compounds **8** and **11**). Compound **11** with a hydroxy group is a much stronger Chk1 inhibitor than compound **10**, bearing a benzyloxy substituent. 1,3,4,6-Tetrahydro-10-hydroxy-7*H*-dipyrrolo[3,4-*a*:3,4-*c*]carbazole-1,3,4,6-tetraone **11** is the most efficient Chk1 inhibitor in this bis-imide series with an IC_{50} value of 2 nM.

A methyloxy substituent in the 11 position is detrimental to Chk1 inhibition. Unfortunately, some compounds, 11-OBn, 10-F, 10-Cl, 10-I, and 10-CO_2CH_3, could not be tested due to their insolubility.

All of the compounds bearing modified imides (compounds **13**–**21**) were poor Chk1 inhibitors except compound **13**, in which the upper imide is not substituted. However, compound **13** was a much weaker Chk1 inhibitor than compound **A**, suggesting that substitution of the lower imide nitrogen was also detrimental to Chk1 inhibition. Replacement of the upper imide function by an anhydride, or substitution of the upper imide nitrogen with a methyl or a *N*,*N*-diethylaminoethyl group abolished the Chk1 inhibitory properties. The aza bis-imide analogue **22** presented a Chk1 inhibitory activity in the micromolar range. However, the replacement of the indole moiety by a 7-azaindole strongly weakened the activity toward Chk1 (compare compounds **22** and **A**). As observed in the non-aza series, in the aza series, the substitution of the upper imide nitrogen with a methyl group completely abolished the Chk1 inhibitory properties.

Bis-imide analogues bearing a sugar part in the non-aza and aza series were synthesized. The sugar moiety was expected to increase compound solubility by forming supplementary hydrogen bonds in the ATP binding pocket, as observed in the crystal structure of UCN-01/Chk1 complex (Zhao et al., 2002). However, the glycosylation, except for hydrochloride **28**, did not improve significantly the solubility. In the non-aza series (compounds **24**–**28**), compound **24** without modifications of the glucosyl unit was the most efficient Chk1 inhibitor. The sugar moiety did not enhance Chk1 inhibition (compare compounds **24** and **A**). The replacement of hydroxy functions on the sugar by acetoxy groups strongly weakened the Chk1 inhibitory potency. These ester functions were introduced to allow a better cell penetration as reported for indigo derivatives (Wang et al., 2003). They are expected to be cleaved inside the cells by enzymes such as esterases to give the hydroxy functions. But the weaker cytotoxicities of compound **25** toward the tumor cell line tested compared to those of compound **24**, suggest that

compound **25** does not function as a prodrug for compound **24**. Chloro, azido and amino groups as R_5 did not improve Chk1 inhibitory activity (compare the IC_{50} values for compounds **26** and **28** with that of compound **24**). In the aza series, compared with its non-aza analogue **24**, compound **29** has lost the capacity to inhibit Chk1. As previously observed with compounds bearing a methyl group at the upper imide nitrogen, compound **30** showed low activity against Chk1. Interestingly the most efficient compound in the aza series bearing a sugar unit is compound **31**, substituted at the upper imide nitrogen with a hydroxymethyl group, exhibiting a Chk1 inhibitory activity in the micromolar range.

3.2. Inhibitory activities of compounds **A**, **5**, *and* **11** *toward a panel of other kinases*

To evaluate the selectivity of this family of compounds, the inhibitory activities of bis-imide **A** toward various other kinases (AMPK, CAMKII, CKI, FGFR3, GSK3, LCK, MAPK1, MAPKAPK2, P70S6K, PKA, PKCβ, PKCα, PKCε reported in Table 2) were evaluated. Interestingly, the strongest inhibition was found toward Chk1, which confirms the specificity of this compound on this panel. Moreover, the inhibitory activities of compounds **A**, **5** and **11** toward Src tyrosine kinase were examined. The percentages of Src inhibition were determined at a given drug concentration (10 μM for **A** and 4 μM for **5** and **11**) (Table 3). In contrast with their high inhibitory activities toward Chk1, these three compounds did not exhibit strong inhibitory properties toward Src.

3.3. In vitro antiproliferative activities against five tumor cell lines: murine L1210, human DU145, A549, HCT116 and HT29

The antiproliferative activities against cell lines in vitro were evaluated. The IC_{50} values in μM are given in Table 1. It can be noticed that most of the tested compounds were not strongly cytotoxic. For example, the cytotoxicities of compound **A**, a potent Chk1 inhibitor with an IC_{50} value of 17 nM, and

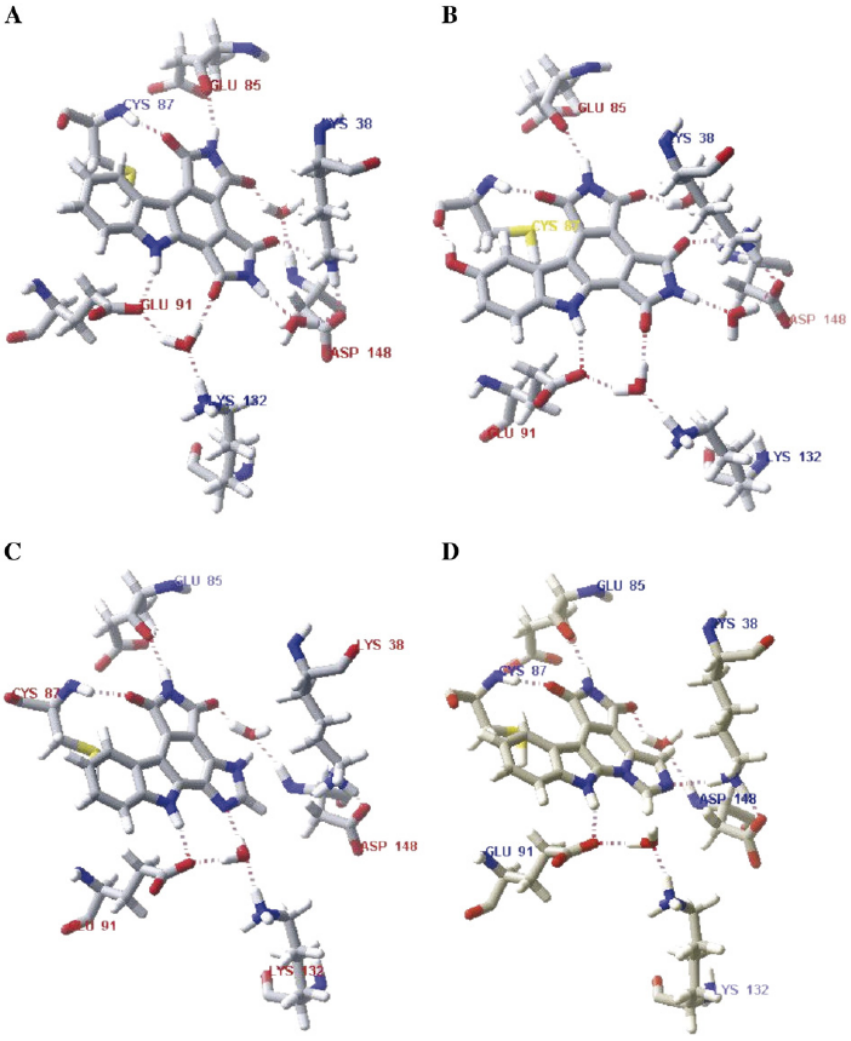

Fig. 4. Molecular modelling of bis-imides **A** (A) and **11** (B), granulatimide (C), and isogranulatimide (D) in the ATP binding site of Chk1.

compound **11** (IC_{50} toward Chk1: 2 nM) toward the tumor cell lines tested are moderate. The highest cytotoxicities were observed for compounds **B**, **4**, **5** bearing 10-CH_3, 9-Cl and 9-Br substituents on the indole moiety, respectively.

4. Discussion

From our results, it appears that some bis-imides are considerably stronger Chk1 inhibitors than their parent natural compounds granulatimide and isogranulatimide. That could be explained by more affinity for the enzyme. To get an insight into the mode of binding of our bis-imides compared with granulatimide and isogranulatimide, molecular modelling of granulatimide, isogranulatimide and our bis-imides **A** and **11** was carried out into the ATP binding pocket of Chk1 (Fig. 4). With bis-imide **A** (Fig. 4A), seven hydrogen bonds were observed. In addition to the two fundamental hydrogen bonds between the carbonyl on the left and the imide NH of the E heterocycle with Cys^{87} and Glu^{85} respectively, hydrogen bonds were also observed between the indole NH and the carboxylate of the side chain of Glu^{91}, and between the upper carbonyl of the D heterocycle and the amine of the side chain of Lys^{38}. Moreover three hydrogen bonds are formed via a water molecule: between the carbonyl on the left of the E heterocycle and the NH of Asp^{148}, between the imide NH of the D heterocycle and the carboxylate of the side chain of Asp^{148}, and between the lower carbonyl of the D heterocycle and the amine of the side chain of Lys^{132} and the carboxylate of the side chain of Glu^{91}. The important hydrogen bond network is in agreement with the strong inhibitory activity of bis-imide **A** toward Chk1 (IC_{50} value: 20 nM). The same hydrogen bond network was observed with bis-imide **11** (Fig. 4B), with an additional hydrogen bond between the 10-OH and the carbonyl of Cys^{87} which could account for the stronger Chk1 inhibitory activity of compound **11** (2 nM). With granulatimide, molecular modelling (Fig. 4C) showed a similar hydrogen bond net than that observed for bis-imide A, except with the D heterocycle for which only one hydrogen bond is formed via the intermediate of a water molecule between the lower nitrogen atom and the amine of the side chain of Lys^{132} and the carboxylate of the side chain of Glu^{91}. This could account for the lower Chk1 inhibitory activity of granulatimide. With isogranulatimide (Fig. 4D) which also showed a similar mode of binding, a hydrogen bond is observed between the nitrogen atom of the imidazole and the amino group of Lys^{38}. The crystal structure of isogranulatimide/Chk1 complex shows differences with what was obtained by molecular modelling. Three hydrogen bonds are observed between the ATP binding site of Chk1 and the drug: the two classical hydrogen bond with the imide heterocycle E and another one between one of the nitrogen atoms of the imidazole and the carboxylic acid of the side chain of Glu^{17} (Jiang et al., 2004).

Interestingly, the two hydrogen bonds formed with the NH and with the carbonyl on the left of the E heterocycle are always found in the crystal structures of Chk1 inhibitors in complex with Chk1. These hydrogen bonds are also observed between the lactam NH and CO of UCN-01 and staurosporine with the ATP binding site of the CDK2 kinase (Gray et al., 1999). Accordingly, the poor Chk1 inhibitory activities of compounds, in which the upper imide nitrogen is substituted, are not surprising.

Concerning the cytotoxicities, except for compounds **B**, **4** and **5** the cytotoxicities toward the tumor cell lines tested are moderate. Chk1 inhibitors are not expected to be cytotoxic by themselves. Theoretically, Chk1 inhibitors are expected to be cytotoxic in the presence of a DNA-damaging agent. However, the low cytotoxicities of the most potent Chk1 inhibitor in this series could be explained by their possible instability inside the cells. This family of compounds seems to be sensitive to nucleophilic reagents and their sensitivity seems to vary according to the substituents on the indole unit. It could be possible that the cytotoxicity observed is due to breakdown products rather than to the presumed compounds. This hypothesis is currently under investigation.

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