Synthesis and super potent anticancer activity of tubulysins carrying non-hydrolysable *N*-substituents on tubuvaline

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This article is dedicated to the memory of Professor Pierfrancesco Bravo.

Abstract: Synthetic tubulysins **24a-m**, having non-hydrolysable *N*-substituents on tubuvaline (Tuv), were obtained in high purity and good overall yields using a multi-step synthesis. Key step was the formation of differently *N*-substituted Ile-Tuv fragments **10** *via* aza-Michael reaction of azido-Ile derivatives **8** with the α,β-unsaturated oxo-thiazole **5**. A SAR study using a panel of human tumor cell lines showed strong anti-proliferative activity for all compounds **24a-m**, with IC_{50} values in the sub-nanomolar range, which were distinctly lower than those of Tubulysin A, vinorelbine, and paclitaxel. Furthermore, **24a-m** were able to overcome cross-resistance to paclitaxel and vinorelbine in two tumor cell lines with acquired resistance to doxorubicin. Compounds **24e** and **24g** were selected as leads to evaluate their mechanism of action. *In vitro* assays showed that both **24e** and **24g** interfere with tubulin polymerization in a vinca alkaloid-like manner and prevent paclitaxel-induced assembly of tubulin polymers. Both compounds exerted antimitotic activity and induced apoptosis in cancer cells at very low concentrations. Compound **24e** also exhibited potent antitumor activity at well tolerated doses on *in vivo* models of diffuse malignant peritoneal mesothelioma, such as MESOII peritoneal mesothelioma xenografts, whose growth was not significantly affected by vinorelbine. These results indicate that synthetic tubulysins **24** could be used as standalone chemotherapeutic agents in difficult-to-treat cancers.

Introduction

Natural tubulysins **1** (Fig. 1) belong to a family of tetrapeptides isolated from myxobacterial culture extracts. They incorporate L-isoleucine (Ile) and three non-proteinogenic amino-acids: *N*-methyl-D-pipecolic acid (Mep), tubuvaline (Tuv), and either tubutyrosine (Tut) or tubuphenylalanine (Tup).[1] Tubulysins, which feature seven stereogenic centers and a peculiar *N,O*-acetal group on Tuv (Table 1), received a great deal of interest since their discovery, principally for their potent cytotoxicity (IC_{50} values in the pico-/nano-molar range) against various human tumor cell lines, such as KB-3.1 and KB-V1 (cervix),[1-3] HEK293T (kidney),[2] U-2 OS (bone),[2] SW-480 and HCT-116 (colon),[2,4,5] K-562 and HL-60 (leukemia),[1,2,5,6] A549 (lung),[2,5] 1A9 (ovarian),[7] MCF-7 and MDA-MB-231 (breast).[5,7,8]

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Tubulysins are produced by several myxobacteria strains.[1,3] Although their biosynthetic pathway has not been fully elucidated yet, it has been shown that tubulysins are produced by a mixed nonribosomal peptide synthetase-polyketide synthase system.[9,10] All the biosynthetic routes hitherto investigated for producing natural tubulysins evidenced serious limitations, principally the low yields. Various synthetic approaches have thus been investigated for producing tubulysins in amounts sufficient for performing preclinical or clinical studies. Synthetic strategies based on the coupling of the four amino-acids evidenced the critical issue of introducing the tertiary amide-*N,O*-acetal on the Tuv residue, along with its acid- and base-lability. Although solutions have been proposed in this sense,[11-13] to our knowledge none of the published syntheses has yet to be used to secure sufficient amounts of tubulysins for clinical development. Alternative strategies were focused on the production of simplified analogues *via* replacement of the *N,O*-acetal moiety with hydrogen (tubulysin U and V and their analogues)[7,14-17] or alkyl

and -CH$_2$-O-alkyl groups.[18] A further strategy relied on the incorporation of a stable retro-amide moiety carrying a dipeptoid residue leading to "tubugi" derivatives [19].[19] Pre-tubulysins, having structurally simplified Tuv fragments lacking the OAc group, have been also proposed.[20] In general, all these modifications evidenced a much lower antiproliferative activity of the synthetic derivatives relative to the most potent natural tubulysins. Ultimately, IC$_{50}$ (concentrations inhibiting cell growth by 50%) values at least two order of magnitude higher were consistently observed upon N,O-acetal ester replacement by a hydrogen atom.[1,15]
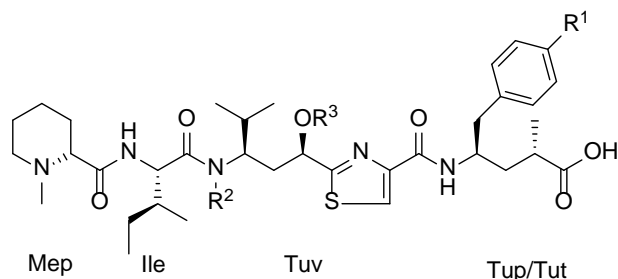

Figure 1. Chemical structures of natural tubulysins **1**. R^1, R^2, and R^3: various substituents (see Table 1).

		Table 1. Natural tubulysins 1.		
Tubulysin	R^1	R^2	R^3
A	OH	CH$_2$OC(O)CH$_2$CH(CH$_3$)$_2$	C(O)CH$_3$
B	OH	CH$_2$OC(O)CH$_2$CH$_2$CH$_3$	C(O)CH$_3$
C	OH	CH$_2$OC(O)CH$_2$CH$_3$	C(O)CH$_3$
D	H	CH$_2$OC(O)CH$_2$CH(CH$_3$)$_2$	C(O)CH$_3$
E	H	CH$_2$OC(O)CH$_2$CH$_2$CH$_3$	C(O)CH$_3$
F	H	CH$_2$OC(O)CH$_2$CH$_3$	C(O)CH$_3$
G	OH	CH$_2$OC(O)CH=C(CH$_3$)$_2$	C(O)CH$_3$
H	H	CH$_2$OC(O)CH$_3$	C(O)CH$_3$
I	OH	CH$_2$OC(O)CH$_3$	C(O)CH$_3$
U	H	H	C(O)CH$_3$
V	H	H	H
X	OH	H	C(O)CH$_3$
Z	OH	H	C(O)CH$_3$

Total synthesis of modified tubulysins combined with structure-activity relationship studies allowed to define key structural parameters related to tubulysin cytotoxicity.[9,21-24] The Tuv carbon atoms stereochemistry as well as the presence of both isopropyl and acetyl group in the Tup fragment are recognised as the most important features for maximizing cytotoxicity. D-configuration of the stereocentre and the Mep N-alkyl substitution are other sensitive structural parameters. Minor effects on cytotoxicity have been instead associated with N,O-acetal moiety replacement with carbon-chains, thiazole ring substitution (although within few tested alternative aromatic or heteroaromatic rings), Tup stereochemistry, and the size of the aza-cycloalkyl moiety.[9,21-26] Moreover, a C$_6$H$_4$-4-OH instead of a C$_6$H$_5$ residue in the Tup fragment induced a weak cytotoxicity drop, as a likely consequence of an overall lipophilicity decrease.[9]

Although both natural and synthetic tubulysins showed strong anti-cancer potential, to our knowledge unconjugated tubulysin derivatives have never been successfully used *in vivo*, due to their reported extremely narrow therapeutic windows. De facto, the tubulysin dosage and formulation used so far in *in vivo* experiments either induced animal death or showed no significant therapeutic effect.[27]

Very interesting results have been conversely achieved when natural tubulysin derivatives were conjugated with folic acid, DUPA or linear β-cyclodextrin and polyethylene glycol (CDP) copolymers.[27-31] Clinical trials are currently in progress on conjugates of Tubulysin B hydrazides with folic acid or DUPA. Moreover, pre-clinical *in vivo* assays evidenced anti-tumor activity of CDP-TubA nanoparticles based on a thiol derivative of tubulysin A linked to CDP.[27] Tubulysin B has also been tested as payload in cholecystokin 2 receptors (CCK2R) small molecule ligand conjugates.[32] Tubulysin derivatives have been also conjugated to dendrimers as macromolecular drug carriers.[33]

Recently, an antibody drug conjugate (ADC) based on a synthetic tubulysin analogue and HER2/neu antibody trastuzumab (Herceptin®) has been tested in an animal model of HER2/neu receptor expressing tumor.[34] This ADC inhibited tumor growth in a nude mice bearing trastuzumab-sensitive N87 tumor model and showed a dose-response effectiveness equivalent to that induced by 15 mg/kg of ado-trastuzumab emtansine ADC (Kadcyla®) at the dose of 60

mg/kg.[34] More recently, other synthetic analogues of natural tubulysins have also been successfully tested as payloads in HER2-based ADCs.[35]

To identify novel synthetic tubulysin derivatives with improved anti-tumor activity, with the aim of using them as cytotoxic payloads in ADCs, our group patented an innovative and scalable synthetic procedure to prepare a specific class of analogues with enhanced cytotoxicity relative to that of natural tubulysins.[36] Very recently, we reported on the cytotoxicity (IC$_{50}$ in the pM range) and mechanism of action of a first lead analogue **24b** (Table 2), or KEMTUB010, having a non-hydrolysable benzyl group on the *N*-Tuv fragment and a Tup residue incorporating a C$_6$H$_4$-4-F group.[8]

Here we provide a full account of our studies on the synthesis, structure activity relationship (SAR) and *in vitro* anti-cancer activity of the super-potent tubulysin derivatives **24a-m**. Based on the SAR study on cells, a lead tubulysin derivative (compound **24e**) was then assayed in animal models.

Results and Discussion

Synthesis of tubulysin derivatives

Our aim was to increase both lipophilicity and chemical stability of the novel tubulysin analogues relative to both natural tubulysins **1** and known synthetic analogues. We decided to replace the *N*,*O*-acetal group on the *N*-Tuv fragment of **1** with non-hydrolysable and variably hydrophobic carbon-substituents: benzyl, phenyl, -CH$_2$-cyclo(etero)alkyls, -CH$_2$-eteroaryls, -CH$_2$-CH$_2$-O-CH$_3$.

To further modulate the lipophilicity of these derivatives, we decided to include in the SAR study analogues carrying either a phenyl or a *p*-fluorophenyl group on the Tup fragment. As suggested by previous SAR studies,[9,21,22] the configuration of the seven stereogenic centers of natural tubulysins **1** was maintained. Moreover, both isopropyl and acetyl groups in the Tuv fragment, as well as the *N*-methyl substitution of the Mep residue were also maintained in all the novel compounds. Table 2 shows the chemical structure of all the tubulysin derivatives **24a-m** synthesized in this work.

The synthesis of **24a-m** was achieved by assembling of three fragments: Ile-Tuv (**11**, Scheme 2), Tup (**19**, Scheme 4) and Mep. To obtain the Ile-Tuv fragment we started from the synthesis of the thiazole ring according to previously reported procedures (Scheme 1).[15] L-Cysteine ethyl ester hydrochloride **2** was condensed with methylglyoxal to give the intermediate 2-acetyl-4-ethoxycarbonylthiazolidine **3**, which was quickly dissolved in CH$_3$CN and oxidized with MnO$_2$ at 65 °C to provide 2-acethylthiazole **4**. Aldol condensation was carried out with thiazole **4** and isobutyraldehyde using catalytic TiCl$_4$ as catalyst, affording the α,β unsaturated thiazolyl ketone **5**.
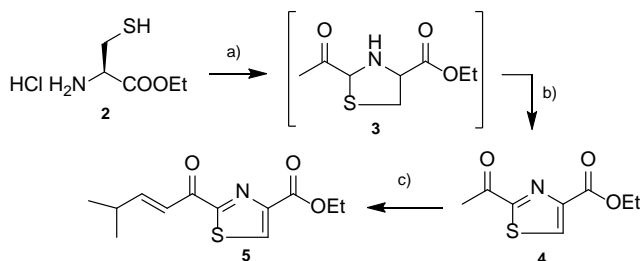
Scheme 1. Synthesis of the α,β unsaturated thiazolyl ketone **5**. Reagents and conditions: (a) methylglyoxal, NaHCO$_3$, EtOH-H$_2$O (1:1), overnight; (b) MnO$_2$, MeCN, 65 °C, overnight (52% over two steps); (c) isobutyraldehyde, TiCl$_4$, Et$_3$N, anhydrous THF, from -78 °C to rt (70%).

Next, a series of amines **6** was reacted with the enantiomerically pure azido acyl chloride derivative **7** affording the azido derivatives **8a-i** with yields ranging from 80 to 99% (Scheme 2).

Aza-Michael reaction of azido derivatives **8a-i** with α,β unsaturated thiazolyl ketone **5** (Scheme 2) afforded β-amino ketones **9a-i**, thus enabling the incorporation of the different *N*-substituents into the Ile-Tuv fragment. Derivatives **9a-i** were obtained as diastereoisomeric mixtures, in variable ratios depending on the R^1 substituent of the azido derivatives **8**. Moderate diastereoselectivities were observed for **9b,c,d** (d.r. 70:30, 75:25, 73:27), with a drop of diastereoselectivity for **9a,g,h,i** (d.r. 68:32, 63:37, 66:34, 68:32). No or very low diastereoselectivity was instead observed for **9e,f** (d.r. 50:50, 55:45). To prepare stereochemically pure Tuv fragments, we performed an asymmetric reduction of the carbonyl function using chiral oxazaborolidines (Corey-Bakshi-Shibata, CBS, catalyst). (*S*)-CBS in the presence of BH$_3$•Me$_2$S predominantly reacted with the *Si* face of derivatives **9a-i** to give alcohols **10a-i** and **10'a-i**, which were easily obtained in diastereomerically pure form and high enantiomeric excess by flash chromatography (FC). The last step of Tuv fragment synthesis was carried out only on diastereisomers **10a-i**, having the same configuration as the natural tubulysins, which were submitted to gentle hydrolysis to the corresponding carboxylic acids **11a-i** (Scheme 2).
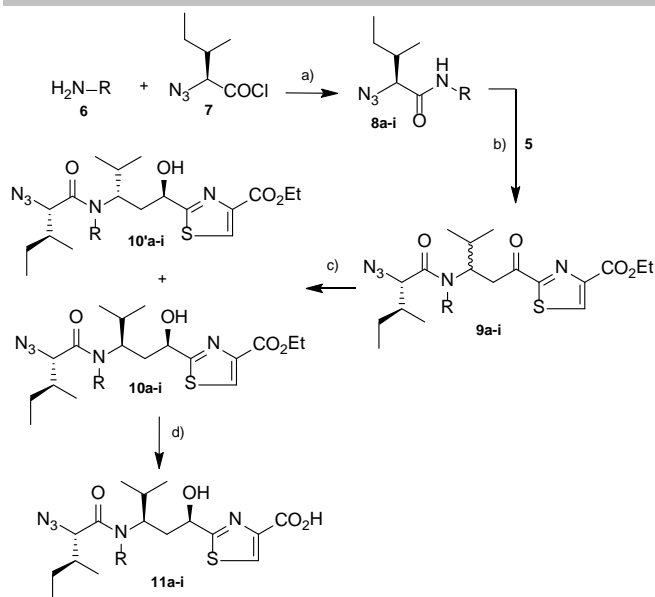
Scheme 2. Synthesis of the azido derivatives **8a-i**. Reagents and conditions: (a) *i*-Pr$_2$EtN, CH$_2$Cl$_2$, 0 °C, 1h, 80 to 99%; (b) KHMDS, anhydrous THF, -78 °C, 1h, overall 15 to 54%; (c) (S)-CBS, anhydrous THF, BH$_3$•Me$_2$S 10M, from 0 °C to rt, overall 32 to 81%; (d) LiOH, THF/H$_2$O 4:1, 12h, rt, 60 to 99%

The synthesis of the Tup fragment was based on the use of (−)-menthol as chiral auxiliary, which strongly facilitates the separation of Tup diastereomers by FC. As previously reported,[21] (−)-menthol was treated with bromoacetyl bromide to give compound **12** (Scheme 3). The latter was converted into the corresponding phosphonium ylide **13** by treatment with triphenylphosphine and subsequently with aqueous NaOH. Methylation of **13** with CH$_3$I gave the key reagent **14**.
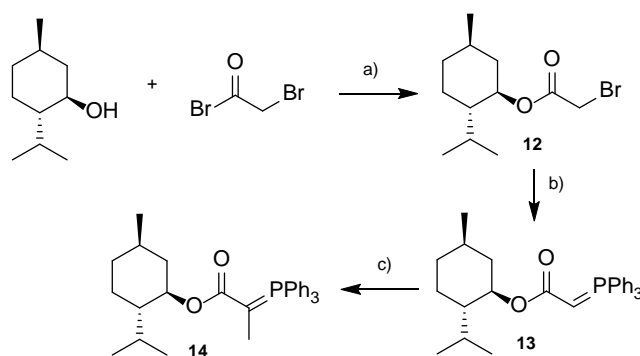

Scheme 3. Synthesis of the phosphonium ylide **14**. Reagents and conditions: (a) Et$_3$N, anhydrous THF, from 0 °C to r.t., 2h; (b) PPh$_3$, THF reflux, 2h, NaOH 0.38N, toluene, rt; (c) MeI, CH$_2$Cl$_2$, from 0 °C to rt, overnight.

Wittig olefination of ylide **14** and aldehydes **16a,b** (Scheme 4), prepared by Dess-Martin periodinane oxidation of the corresponding Boc protected phenyl-alaninols **15a,b**, gave the α,β unsaturated amino esters **17a,b** in good yields. These compounds were hydrogenated using Pd/C (10%) in ethyl acetate to give the Tup fragment precursors **18'a,b** and **18"a,b** as mixtures of diastereoisomers, which were separated by FC (d.r. **18'a/18"a** = 70/30 and d.r. **18'b/18b"** = 80/20). Only diastereomers **18'a,b** – which have the natural Tup stereochemistry - were used for the next steps of the synthesis. The final Tup fragments **19a,b** were obtained using a two steps procedure based on: (i) simultaneous de-protection of both amino and carboxylic functions with 6N HCl at 130 °C to give the corresponding free amino acids as hydrochlorides; (ii) *O*-methylation to give the enantiomerically pure methyl esters **19a,b** (Scheme 4).[37]
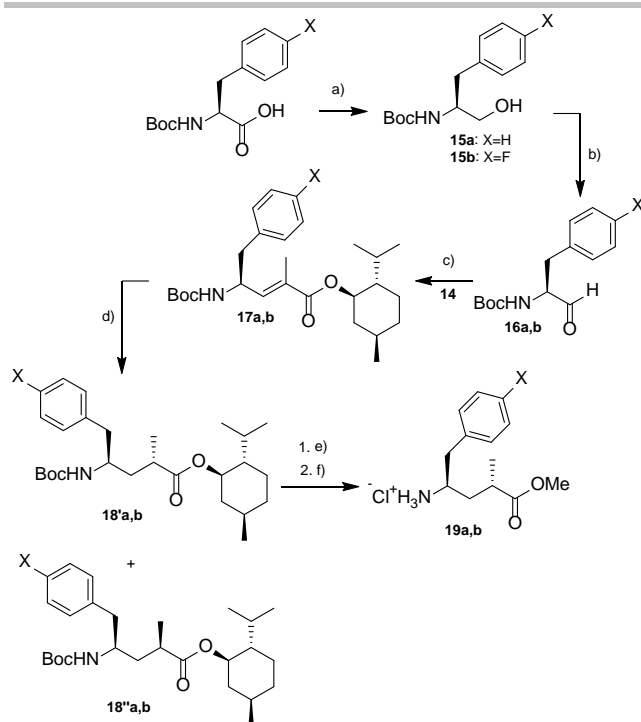
Scheme 4. Synthesis of stereopure 4-amino-2-methyl-5-phenylpentanoic acid **19a** and 4-amino-5-(4-fluorophenyl)-2-methyl-pentanoic acid **19b** methyl esters hydrochlorides. Reagents and conditions: (a) anhydrous THF, 0 °C, ethyl chloroformate, Et$_3$N, 1h, NaBH$_4$ in H$_2$O at 0 °C, 63%; (b) Dess-Martin periodinane, NaHCO$_3$ CH$_2$Cl$_2$, 2h, 98% for **16a**, 99% for **16b**; (c) CH$_2$Cl$_2$, from 0 °C to rt, 2h, 78% for **17a**, 74% for **17b**; (d) H$_2$, Pd/C 10%, EtOAc, overnight, overall 87% for **18a**, overall 97% for **18b**; (e) HCl 6N, 130 °C, 1h; (f) 2,2-dimethoxypropane, HCl 37% 2µL, MeOH, 50 °C, 12h, 91% for **19a**, 93% for **19b**.

Assembling of the final tubulysin derivatives **24a-m** was achieved using conventional peptide synthesis in solution (Scheme 5). Coupling of the Tuv fragments **11a-i** with Tup methyl ester hydrochloride derivatives **19a,b** afforded the tripeptides **20a-m**. Next, the azide function of compounds **20a-m** was reduced by hydrogenation over Pd/C (10%) to give the amino derivatives **21a-m**. These compounds were not isolated from the reaction mixtures but were directly subjected to coupling with Mep, affording tetrapeptides **22a-m** which were isolated in good yields. The methyl ester function of **22a-m** was hydrolysed with LiOH in THF, followed by TFA treatment at pH=2 to afford compounds **23a-m**, isolated as TFA salts. Finally, stereopure tubulysin derivatives **24a-m** were obtained by acetylation of the hydroxyl function of **23a-m** in Ac$_2$O and subsequent neutralization with pyridine.
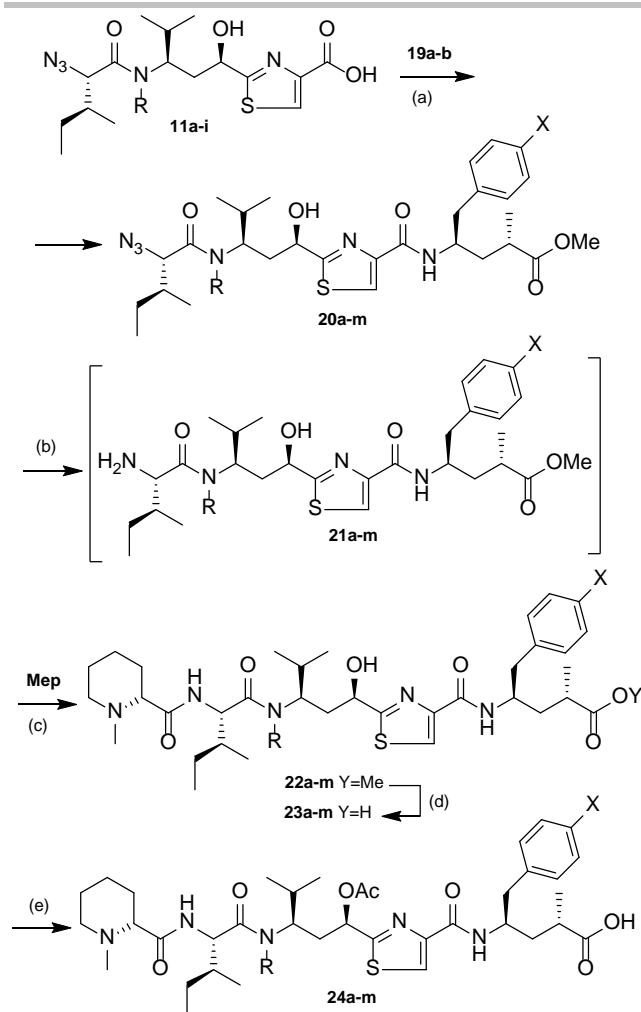
Scheme 5. Synthesis of tubulysin derivatives **24a-m**. Reagents and conditions: (a) HATU, HOAt, Et$_3$N, DMF, rt, 2h, 70 to 98%; (b) H$_2$, Pd/C 10%, MeOH, overnight, 99%; (c) HATU, HOAt, Et$_3$N, CH$_2$Cl$_2$, rt, 4h, 73 to 99%; (d) LiOH 1N, THF, 48h, rt, TFA pH=2, 71 to 99%; (e) Ac$_2$O, pyridine, rt, overnight, 63 to 99%.

In vitro and in vivo assays

All the tubulysin analogues **24a-m** were tested for their antiproliferative activity on human colon cancer cell line HT-29 (Table 2). These compounds were compared to TubA; TubU,[22] and the N-Me tubulysin U derivative Me-TubU (R = Me, X = H in structure **24** in Table 2). All the assayed compounds showed strong cytotoxic activity, with IC$_{50}$ values in the pM range (IC$_{50}$ < 0.28 nM), which were lower than that of the reference natural tubulysin TubA (IC$_{50}$ = 0.75 nM), and significantly lower than those of the synthetic tubulysin derivatives reported so far in the literature (IC$_{50}$ values in the order of nM units). Particularly, **24b**, **24e**, **24g**, **24h**, **24j**, and **24m**, all showed IC$_{50}$ values lower than 0.10 nM, being significantly more cytotoxic than TubA. The same trend and lower IC$_{50}$ values relative to that of TubA were detected also on human ovarian carcinoma cell line A2780 (data not shown).

Table 2. Chemical structure and HT-29 cell cytotoxicity (IC_{50} pmol L^{-1})[a] of the synthetic tubulysin derivatives **24**.

Compound	R	X	HT-29 cell line
24a		H	112±24
24b		F	61±9
24c		H	101±15
24d		H	225±27
24e		H	55±10
24f		F	190±12
24g		H	82±12
24h		F	90±15
24i		F	183±10
24j		F	92±13
24k		F	102±19
24l		H	278±25
24m		F	95±16
TubA	$CH_2OC(O)CH_2CH(CH_3)_2$	OH	752±51
TubU	H	H	3780±125
Me-TubU	CH_3	H	5820±198

[a] Concentration of tested compounds able to inhibit cell growth by 50%.

On this cell line, IC_{50} values of **24a**, **24c**, and **24k** were comparable to those of the most cytotoxic compounds measured on HT-29. In general, amongst the novel series of synthetic tubulysins, no substantial effect on antiproliferative activity was detected upon (i) replacement of hydrogen with fluorine in position 4 of the Tup fragment benzyl moiety of series **24** (X-residue in Table 2), and (ii) the nature of the carbon *N*-substituent of the Tuv residue.

Based on the results of the preliminary screenings, representative selected compounds amongst this series were submitted to further tests by adopting a wider spectrum of tumor cell lines. In particular, the antiproliferative activity of these tubulysin derivatives was evaluated on a panel of human tumor cell lines, including colon (LoVo) and breast (MCF-7) cancer cell lines, their derivatives selected for in vitro acquired resistance to doxorubicin (DX) and showing cross-resistance to taxanes and vinca alkaloids (LoVo/DX; MCF-7/DX),[38,39] and on two diffuse malignant peritoneal mesothelioma (DMPM)[40] cell lines (STO and MESOII), established in our laboratories.[41] Exposure of cancer cells to increasing concentrations of tested compounds resulted in a markedly higher cytotoxic effect compared to those of two

microtubule-interacting agents with opposite mechanism of action (i.e. paclitaxel and the vinca alkaloid vinorelbine), used as reference compounds (Table 3).

The cytotoxic effect of all the assayed tubulysin derivatives was consistently observed across all tested cancer cell lines, including those characterized by acquired drug resistance.[39] Reference compounds paclitaxel and vinorelbine showed lower antiproliferative activity compared to all the novel tubulysin derivatives, particularly relative to **24a**, **24b**, **24c**, **24e**, **24g**, and **24h**. In general, these compounds showed IC_{50} values 1-2 order of magnitude lower than vinorelbine on MESOII, STO, LoVo, and MCF-7 cell lines. The comparison was even more favorable for these tubulysins if paclitaxel was considered as reference compound. IC_{50} value of **24b** – or KEMTUB010 - on MCF-7 cell line was in accordance with that previously determined for the same compound.[37] Amongst the novel tubulysin derivatives, **24e** and **24j** showed respectively the lowest and the highest IC_{50} values throughout the entire panel of cell lines tested.

The superior cytotoxic activity of these tubulysin derivatives compared to the reference compounds was confirmed *in vitro* by using cell lines with acquired resistance to doxorubicin. In fact, the tubulysin derivatives **24** showed IC_{50} values at least 23 and 100 fold lower than vinorelbine and paclitaxel, respectively, on LoVo/DX cell line. Compound **24j** – the less potent compound in the series - was 15 and 3 fold more active than vinorelbine and paclitaxel, respectively, on MCF-7/DX cell line. However, the cytotoxicity of all the other tubulysin derivatives **24** on this cell line was significantly higher (IC_{50} values 1-2 orders of magnitude lower than **24j**).

Table 3. Cytotoxic activity of tubulysin derivatives (IC_{50} nmol L^{-1})[a]								
Compound	**Cell Line**							
	MESOII	*STO*	*LoVo*	*LoVo/DX*	*RI[b]*	*MCF-7*	*MCF-7/DX*	*RI[b]*
Paclitaxel	13.65±0.05	11.9±5.5	313±66	11700±1100	37.3	32.4±2.3	307.2±124.7	9.5
Vinorelbine	5.65±2.65	4.8±0.6	98.2±47.7	2355±472	23.9	7.3±2.7	65.4±10.2	8.9
24a	0.215±0.05	0.22±0.07	1.87±1.86	1.35±0.78	0.8	0.37 ± 0.38	0.725 ± 0.39	1.94
24b	0.5±0.003	0.525±0.07	5.4±2.7	4.5±2	0.8	0.32 ± 0.035	1.45 ± 0.07	4.53
24c	0.25±0.07	0.11±0.12	2.5±0.7	1.55±0.07	0.62	0.425 ± 0.3	0.535 ± 0.2	1.25
24e	0.036±0.003	0.019±0.004	0.7±0.4	2.1±0.3	3	0.44±0.22	0.41±0.07	0.93
24g	0.31±0.15	0.24±0.08	3.1±1.2	1.4±0.2	0.45	1.56±0.54	0.98±0.41	0.63
24h	0.47±0.11	0.37±0.14	1.9±1.1	10.5±4.9	5.5	0.74±0.03	1.08±0.39	1.45
24j	6.5±3.5	2.8±1.1	14.2±7.1	~100	7	4.77±0.035	22.2±4.9	4.65
24k	1.97±0.23	1.60±0.61	7.5±4.5	60.9±10	8.12	2.75±0.26	2.97±1.6	1.08
24m	1.08±0.49	0.94±0.62	4.2±2.8	18.1±2	4.3	1.93±0.57	5.71±3.63	2.96

[a] Concentration of tested compounds able to inhibit cell growth by 50%; [b] Resistance index has been defined as the ratio between the IC_{50} values observed in resistant (LoVo/DX and MCF-7/DX) and wild-type (LoVo and MCF-7) cells.

It is important to note that the resistance index (RI), defined as the ratio between the IC_{50} values observed in resistant (LoVo/DX and MCF-7/DX) and wild-type (LoVo and MCF-7) cells, was markedly lower for all tubulysin derivatives ($RI_{LoVo/DX}$ <8; $RI_{MCF-7/DX}$ <5) compared to paclitaxel ($RI_{LoVo/DX}$: 37.3; $RI_{MCF-7/DX}$: 9.5) and vinorelbine ($RI_{LoVo/DX}$: 23.9; $RI_{MCF-7/DX}$: 9.0) (Table 3), thus suggesting that they may be poor substrates for the drug efflux pumps.

The potent activity of these tubulysin derivatives was also confirmed for a selected subclass on additional cancer cell lines, including N87 (gastric carcinoma), BT474 and SkBr3 (breast cancer) (Supporting Information, Table S1). Especially in the two breast cancer cell lines, the novel compounds showed significantly higher cytotoxic activity relative to TubA, with IC_{50} values at least one order of magnitude lower than those of the reference natural analogue.

Compounds **24e** and **24g** were selected as lead compounds for their strong cytotoxic activity and lower RI values, respectively (Table 3). Their effects on microtubule assembly were investigated as the first step of the study on their mechanism of action. Exposure of DMPM cells to either agents, administered at a concentration corresponding to the specific IC_{80}, did not result in perturbations of the intracellular pools of tubulin, as observed in cells treated with vinorelbine and - oppositely - in cells exposed to paclitaxel (Figures 2A and S1, Supporting Information). Similar results were obtained in wild-type (LoVo) and resistant (LoVo/DX) colon cancer cell lines (Supporting Information, Fig. S2).
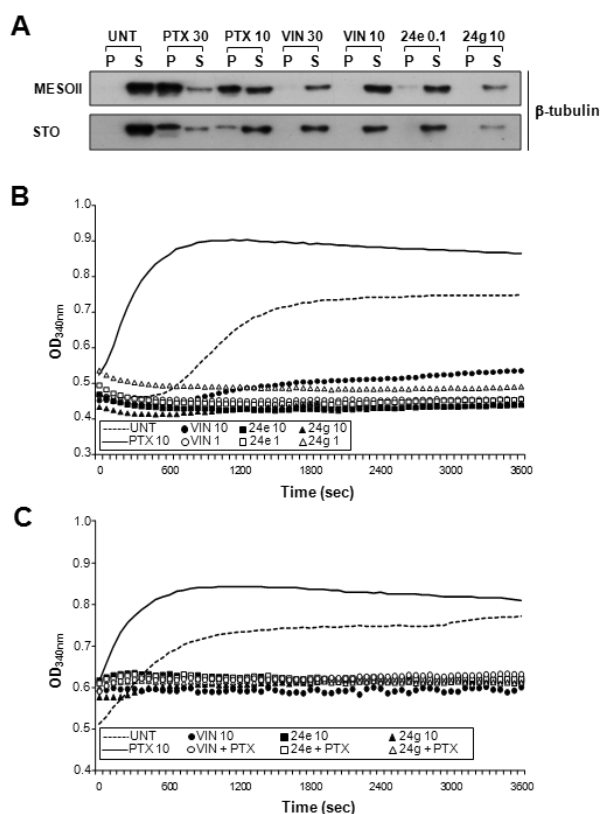
Figure 2. Tubulysin derivatives alter tubulin polymerization according to a vinca alkaloid-like mechanism of action. A) Representative immunoblotting showing the effect on polymerized (P) and soluble (S) fractions of tubulin from DMPM cells upon a 24-h exposure to the different tested agents at the indicated concentrations (nmol L^{-1}); B) Test-tube assessment of tubulin polymerization in the presence of paclitaxel (PTX), vinorelbine (VIN), **24e** and **24g**; used at the indicated concentrations (µmol L^{-1}); C) Effect of tubulysin derivatives and vinorelbine on PTX-induced tubulin polymerization. A representative experiment of two is shown. UNT: untreated samples.

The direct interference of **24e** and **24g** with tubulin polymerization was further evaluated by measuring the GTP-induced assembly of purified tubulin monomers using a test-tube assay. Tubulin polymerization was monitored over time (0-60 min) by measuring the changes in solution turbidity at 340 nm. Results showed that tubulin polymerization was markedly inhibited in samples treated with **24e** or **24g** (1 and 10 µmol L^{-1}, V_{max}: 10±2 mOD/min) compared to untreated controls (V_{max}: 23±1 mOD/min) (Fig 3B). Such an effect, which was similar to that observed in samples treated with equimolar concentration of vinorelbine (V_{max}: 9±2 mOD/min), occurred soon after a 5-min exposure to each agent and persisted over the time course of the experiment. As expected, paclitaxel induced a pronounced increase in the polymerization rate (V_{max}: 62±2 mOD/min) of tubulin compared to controls or samples treated with tubulysin derivative and vinorelbine (Fig. 2B). Such a paclitaxel-induced increase in the tubulin polymerization was remarkably affected by tubulysin derivatives, as suggested by the complete inhibition of the reaction in samples concomitantly treated with paclitaxel and **24e** or **24g** or vinorelbine (Fig. 2C). Indeed, a ~7-fold decrease in the V_{max} was observed in paclitaxel-treated cells in the presence of tubulysin derivates or vinorelbine compared to taxane alone (~8±3 mOD/min and 55±5 mOD/min, respectively). These findings further corroborate our previous observations indicating that tubulysin compounds show a typical vinca alkaloid-like mechanism of action on tubulin dynamics.

Since mitotic arrest is a hallmark of agents able to interfere with microtubule assembly, the capability of **24e** and **24g** to interfere with the progression of cells through the M-phase of the cell cycle was investigated. Specifically, a 24-h exposure of DMPM cells (Fig. 3A) to equitoxic concentrations of **24e** and **24g** (10 and 0.1 nmol L^{-1}, respectively) resulted in a remarkable accumulation of cells (~70%) in the G$_2$/M phase of the cell cycle, similarly to what observed upon exposure to equitoxic amount (30 nmol L^{-1}) of vinorelbine or paclitaxel (Fig. 3A). Furthermore, the analysis of the expression levels of factors known to be involved in the mitotic arrest showed a marked accumulation of mitosis-specific phosphorylated epitopes recognized by MPM-2 antibody (Fig 3B), that was paralleled by a marked up-regulation of cyclin B (Fig. 3B), a factor that plays a pivotal role in the control of G$_2$/M cell cycle transition.[42]

In addition, fluorescence microscopy analysis of cells stained for MPM-2 showed the presence of 50% of mitotic cells within the overall cell population upon a 24-h exposure to equitoxic amounts of **24e**, **24g**, vinorelbine or paclitaxel (Fig. 3C). Finally, the assessment of apoptosis by TUNEL assay showed comparable amounts of apoptotic cells, upon exposure to equitoxic doses of tested derivatives and reference compounds (Fig. 3C). Similar results were obtained in LoVo and LoVo/DX colon cancer cells (Supporting Information, Fig. S3). Overall, these findings indicate that, similarly to

vinorelbine and paclitaxel, the tubulysin derivatives induced mitotic arrest and apoptosis in cancer cells of different histological origin, at much lower concentrations than those required to obtain comparable effects by conventional microtubule-interacting agents. These results confirm the preliminary data we have previously published on the effects induced by KEMTUB010 (**24b**) in a panel of breast cancer cells, particularly in MCF-7 and MDAMB231 (MDA231) cell lines.[8]
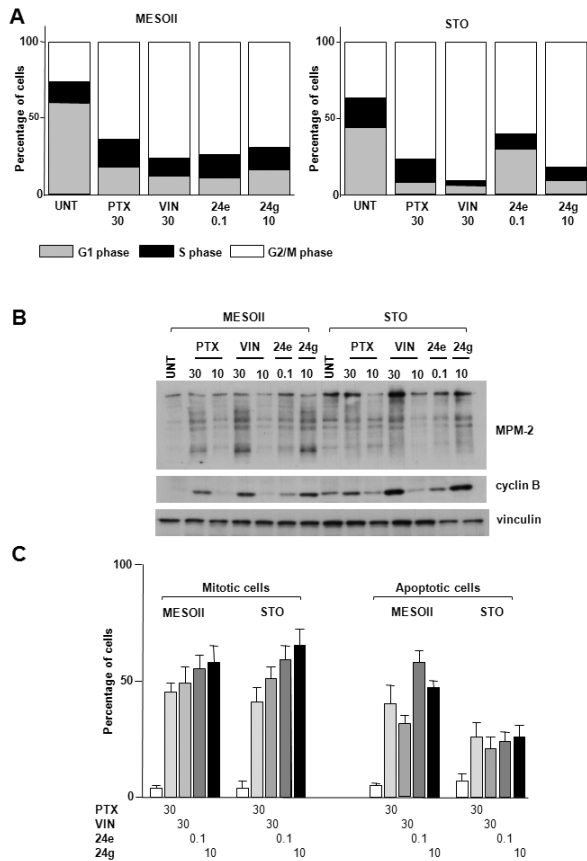

Figure 3. A 24-h exposure to tested compounds induced mitotic arrest and apoptosis in DMPM cells. A) Flow cytometric analysis of the cell cycle distribution in untreated cells (UNT) and in cells exposed for 24 h to paclitaxel (PTX), Vinorelbine (VIN) and the tubulysin derivatives **24e** and **24g** at the indicated concentrations (nmol L^{-1}). Data have been reported as the percentage of cells in the different cell cycle phases and represent mean values from at least three independent experiments; B) Representative western immunoblotting showing the accumulation of mitotic markers in cells following a 24-h exposure to the indicated amounts (nmol L^{-1}) of paclitaxel (PTX), vinorelbine (VIN), **24e** or **24g**. Vinculin was used as a control for equal protein loading. UNT: untreated cells; C) Quantification of data from immunofluorescence and TUNEL assays (see Supporting Information) showing the percentage of mitotic and apoptotic cells upon the exposure to equitoxic concentrations (nmol L^{-1}) of tested compounds, as indicated. Data represent mean values ± s.d. from at least three independent experiments.
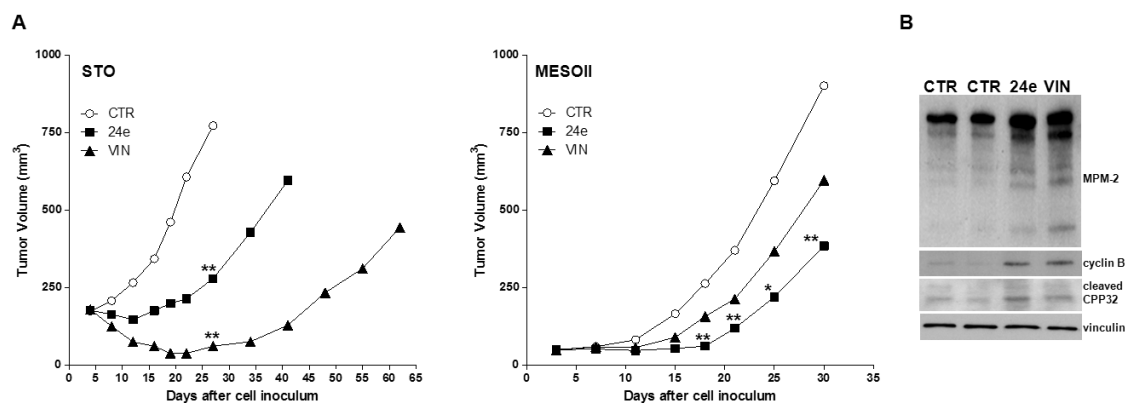
Figure 4. Tubulysin derivatives markedly inhibit DMPM tumor growth *in vivo*. A) DMPM tumor growth curves in untreated mice (CTR) and upon a q4dx4 i.v. administration of **24e** (0.125 mg/kg,) or vinorelbine (VIN, 5 mg/kg). Data have been reported as average TV (mm³). B) Representative western immunoblotting showing the accumulation of MPM-2 and cyclin B and the cleavage of Caspase-3 (CPP32) in MESOII-originated tumors removed from control animals and at the end of treatment with **24e** or vinorelbine (VIN). Vinculin was used as a control for protein loading. CTR: tumors from untreated animals. *P<0.05; **P<0.01.

The antitumor activity of the tubulysin derivative **24e** was then further evaluated on *in vivo* DMPM tumor models, obtained following xenotransplantation of STO and MESOII cells into immunocompromised mice. Aqueous formulations based on Cremophor EL were used for **24e** administration via tail vein intravenous injection. Dosing regimen of **24e** was based on preliminary dose optimization studies in which 0.125 mg/kg administered every four days for four times (q4dx4) showed a significant antitumor activity in the absence of toxic effects (data not shown). The *in vivo* antitumor activity of **24e** was then comparatively evaluated to that of vinorelbine, both administered at their optimal doses (i.e., 0.125 and 5 mg/Kg,[43] respectively) and schedule (q4dx4). Specifically, a significant (*P*<0.01) tumor growth delay was observed in animals treated with **24e** compared to untreated mice in both tumor animal models, with a maximum tumor volume inhibition (TVI) of 64% and 77%, for STO and MESOII, respectively (Table 4 and Fig. 4A).

Table 4. *In vivo* antitumor activity of **24e** and vinorelbine (VIN) on human DMPM cells xenografted into immunocompromised mice.

	Drug	Dose (mg/Kg)	TVI (%)[a]	BWL (%)[b]
STO	**24e**	0.125	64 (27)**	9
	VIN	5	92**	7
MESOII	**24e**	0.125	77 (18)** †	5
	VIN	5	40	3

[a] Percentage of tumor volume inhibition in treated *vs*. control mice. In parentheses the day on which the maximum TVI% was observed; [b] Percentage of body weight loss (BWL) induced by drug treatment. The highest BWL% observed is reported. **P<0.01 *vs*. controls; † P<0.05 *vs*. vinorelbine-treated mice (two-sided Student's t-test).

In particular, although highly significant (*P*<0.01), the antitumor activity of the tubulysin derivative was less pronounced than that exerted by vinorelbine in STO xenografts (Table 4, Fig. 4A). Interestingly, **24e** showed an important antitumor activity also in MESOII xenografts, which, conversely, were not significantly affected in their growth by vinorelbine (Table 4, Fig. 4A). In addition, the compound was well tolerated without any appreciable sign of toxicity and with a restrained effect (<10%) in terms of body weight loss, which was comparable to that observed in animals treated with vinorelbine (Table 4). Finally, the assessment of drug-induced changes in cell cycle-regulated factors in DMPM xenografts showed a marked increase in MPM-2-interacting epitopes, a pronounced accumulation of cyclin B and cleaved Caspase-3 both in **24e-** and vinorelbine-treated mice with respect to controls (Fig. 4B), thus indicating that, similarly to the vinca alkaloid, the tubulysin derivative induced apoptotic cell death upon mitotic arrest also *in vivo* at a lower dose compared to vinorelbine, as observed in the *in vitro* experimental setting.

Conclusions

In conclusion, we have described the first synthetic strategy that can be used to produce routinely over 200 mgs of pure tubulysins **24a-m** per synthetic cycle. To our knowledge, this is the first method that enables the synthesis of a wide range of differently N-Tuv substituted tubulysins on this scale. Novel tubulysin derivatives **24a-m** incorporating non-hydrolysable *N*-substituents on the Tuv fragment displayed IC$_{50}$ values in the pM range on a panel of human tumor cell lines. Their cytotoxicity was significantly superior to that of Tubulysin A, vinorelbine, and paclitaxel, used as reference compounds.

In general, the two main investigated parameters in the SAR study (fluorine or hydrogen atoms in position 4 of Tup benzyl moiety, and *N*-substituent of Tuv) had a minor effect on cytotoxicity, which remained very high throughout. Compounds **24e** and **24g** were selected as lead compounds for their strongest cytotoxic activity and lowest resistance index, respectively, on a panel of cell lines. Surprisingly, differences between the antiproliferative activity of these tubulysins and reference chemotherapeutics were markedly more pronounced on LoVo/DX and MCF-7/DX cell lines characterized by *in vitro* acquired resistance to doxorubicin (DX).

In contrast to paclitaxel and in analogy with vinorelbine, compounds **24e** and **24g** inhibited tubulin polymerization in STO, MESOII, LoVo, and LoVo/DX cell lines. A vinorelbine-like mechanism of action of these tubulysin derivatives was proved by assessing the capacity of **24e** and **24g** to antagonize the paclitaxel tubulin polymerization effect. Moreover, as for vinorelbine and paclitaxel, mitotic arrest and apoptosis in cancer cells were detected upon **24e** and **24g** cell treatments. These tubulysin derivatives exerted the same effect of the reference chemotherapeutics, but at significantly lower concentrations.

In contrast to previously reported studies on natural tubulysins, these analogues showed effective therapeutic windows *in vivo*. In fact, assays in animal models (mice) of DMPM tumors, evidenced a significant antitumor activity of **24e** at the dose of 0.125 mg/kg (maximum tumor volume inhibition of 64% and 77% compared to untreated animals for STO and MESOII, respectively). In STO xenografts, tumor growth delay induced by i.v. administration of **24e** was less marked than that elicited by vinorelbine at the dose of 5 mg/kg. It is important to note that in contrast to vinorelbine (5 mg/kg), **24e** (0.125 mg/kg) was able to inhibit also MESOII xenografts. In addition, the compound was well tolerated with no sign of general toxicity and a restrained effect (<10%) in terms of body weight loss.

These results highlight the potential of these tubulysin derivatives as chemotherapeutics, particularly for treating currently untreatable tumors, such as diffuse malignant peritoneal mesotheliomas. In addition, these highly potent tubulysins may represent very promising payloads in ADCs for targeted cancer therapy.

Experimental Section

Chemistry. All the synthetic procedures, compounds characterizations and copies of ^{1}H, ^{13}C, ^{19}F NMR spectra and MS analyses are included in the Supporting Information.

Biology. Procedures and materials on in vitro assays, cell lines and antiproliferative activity assays, tubulin polymerization assays, in vivo studies, as well as details on methodologies (western immunoblotting flow cytometer analyses, fluorescence microscopy) are described in the Supporting information.

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