Cytotoxicities of three rebeccamycin derivatives in the National Cancer Institute screening of 60 human tumor cell lines

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Among the biologically active indolocarbazoles, rebeccamycin, a microbial metabolite produced by *Saccharothrix aerocolonigenes*, is a well-known topoisomerase (Topo) I poison. In the course of structure–activity relationship studies on rebeccamycin analogs, we have prepared a large number of indolocarbazole derivatives and have shown that, depending on the structural modifications, the cytotoxic effects may be, or not, directly correlated to DNA binding and Topo I inhibition. This suggests that if DNA binding and Topo I play a part in the biological activity of these compounds, other cellular targets might be involved. This paper reports the results of the antiproliferative activities (evaluated in the National Cancer Institute's *in vitro* panel of 60 tumor cell lines) and the results of a COMPARE analysis run with rebeccamycin derivatives to identify other potential biological targets for these compounds.

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Introduction

The properties of indolocarbazole compounds as protein kinase C and topoisomerase (Topo) I inhibitors have been widely studied [1–3]. Among the biologically active indolocarbazoles, rebeccamycin, a microbial metabolite produced by *Saccharothrix aerocolonigenes*, is a well-known Topo I poison [4–6]. In the course of structure–activity relationship studies on rebeccamycin analogs, we have prepared a large number of indolocarbazole derivatives and have shown that, depending on the structural modifications, the cytotoxic effects may be, or not, directly correlated to DNA binding and Topo I inhibition [4,7–11]. To gain an insight into the involvement of Topo I inhibition in the cytotoxicity of rebeccamycin analogs, we have measured the antiproliferative activities of some rebeccamycin derivatives toward P388 and P388CPT5 murine leukemia cells sensitive and resistant to the Topo I inhibitor camptothecin, respectively. In many cases, no direct correlation could be observed between Topo I inhibitory activities and cytotoxicities, suggesting that if DNA binding and Topo I play a part in the biological activity of these compounds, other cellular targets might be involved [8,9,12,13]. Due to the similarity of structures between rebeccamycin and staurosporine, a non-specific kinase inhibitor, various kinases could be suspected to be biological targets for rebeccamycins [14]. In previous studies, we have already shown that some rebeccamycin analogs are able to inhibit kinases such as protein kinase C, protein kinase A, CDK1/cyclin B and CDK5/p25. According to their structures, the rebeccamycin analogs could either interact with DNA and/or inhibit Topo I by stabilizing the cleavage complex and/or inhibit kinases. We have also shown that interaction with DNA and Topo I inhibition can correspond to two separate mechanisms. In particular, the introduction of an amino function at the 6′ position on the sugar moiety enhances the capacity of the drug to interact with DNA, but almost abolishes the poisoning effect on Topo I [10]. Both DNA and Topo I represent two independent targets which can both be used for the development of antiproliferative rebeccamycin derivatives. To improve the solubility and increase the capacity to bind to the cellular target(s), amino acid and peptide substituents have been introduced on the framework of biologically active compounds. This approach has been exploited by a few groups ([15] and references therein) to enhance the biological activities of indolocarbazole derivatives. For that purpose, we have previously prepared, by semi-synthesis from rebeccamycin, two indolocarbazole derivatives NCS 726577 and NCS 726578 bearing a lysine residue on the 2 or 3 positions of the sugar moiety, respectively [15] (Fig. 1). Rebeccamycin was produced in our laboratory by cultures of the bacterial strain *S. aerocolonigenes*. Compared with dechlorinated rebeccamycin NSC 726576, the profiles of cytotoxicity of NCS 726577 and NCS 726578 against L1210 murine leukemia

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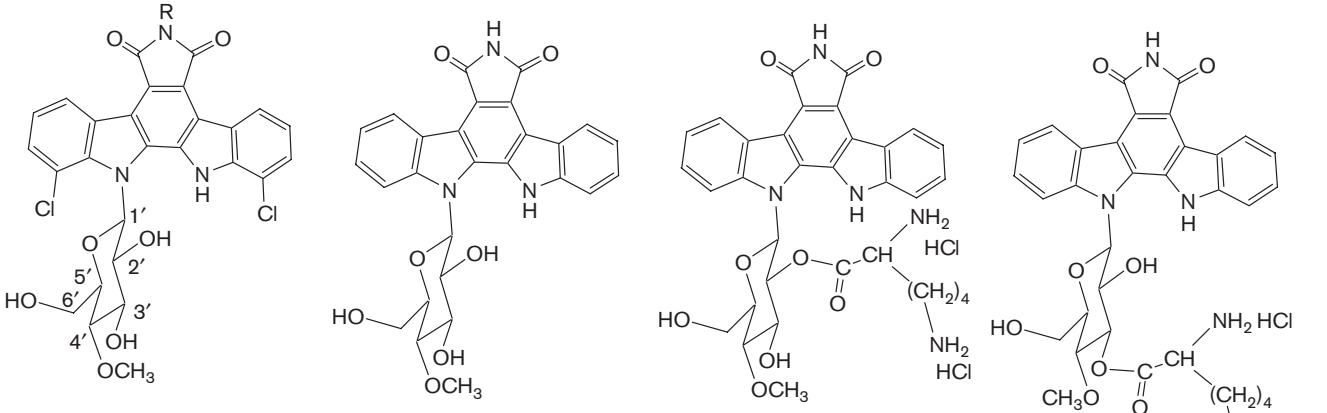
Fig. 1



Indolocarbazole derivatives NSC 726576, NSC 726577, NSC 726578 and NSC 655649 (BMS181176).

cells, human HT29 colon carcinoma, non-small cell lung carcinoma and K-562 leukemia cells was similar [15]. Cell cycle experiments with L1210 leukemia cells revealed that the three compounds induce a massive accumulation of the cells in the G_2/M phase. Since DNA and Topo I have been identified to be biological targets for dechlorinated rebeccamycin [6], the three compounds could share the same mechanism of action, i.e. binding to DNA and poisoning Topo I. The DNA binding constants have been calculated from fluorescence measurements [15]. The variation of the ΔT_m ($T_m^{\text{drug–DNA complex}} - T_m^{\text{DNA alone}}$) showed the following sequence for DNA affinity: rebeccamycin < dechlorinated rebeccamycin NSC 726576 < NCS 726577 = NCS 726578. The antiproliferative activities of these three compounds were evaluated in the National Cancer Institute's *in vitro* panel of 60 tumor cell lines. This paper reports the results of the cytotoxicity assays and the results of a COMPARE analysis run with NSC 726576, NSC 726577 and NSC 726578. The last study has been performed to attempt to determine the biological targets for NSC 726576, NCS 726577 and NCS 726578 by comparison of their *in vitro* cell growth pattern and with those of compounds from the NCI's Standard Agents database and NCI's Synthetics database. The Standard Agents database is a small subset of the Synthetics database and includes compounds that have been clinically developed, with mechanisms at least partially defined.

Materials and methods

Cytotoxicity assays

The drug-screening data was accessed from the existing anticancer screening database generated as described [16]. The sulforhodamine B assay [17] was used to determine the cytotoxicity or growth inhibition of the 60 human tumor cell lines of the NCI panel. Using the seven absorbance measurements [time zero (T_z), control growth (C) and test growth in the presence of drug at the five concentration levels (T_i)], the percentage growth is calculated at each of the drug concentrations levels. Percentage growth inhibition is calculated as:

$[(T_i - T_z)/(C - T_z)] \times 100$ for concentrations for which $T_i \geq T_z$

$[(T_i - T_z)/T_z] \times 100$ for concentrations for which $T_i < T_z$.

Three dose–response parameters are calculated for each experimental agent. Growth inhibition of 50% (GI_{50}) is calculated from $[(T_i - T_z)/(C - T_z)] \times 100 = 50$; GI_{50} measures the growth inhibitory power of the tested agent. The drug concentration resulting in total growth inhibition (TGI) is calculated from $T_i = T_z$; TGI signifies a cytostatic effect. The LC_{50} (concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) indicating a net loss of cells following treatment is calculated from $[(T_i - T_z)/T_z] \times 100 = -50$; LC_{50} signifies a cytotoxic effect. Values are calculated for each of these three parameters if the level of activity is reached; however, if the effect is not reached or is exceeded, the value for that parameter is expressed as greater or less than the maximum or minimum concentration tested.

The mean graph analysis [18]

The mean graph is a pattern created by plotting positive and negative values generated from a set of GI_{50}, TGI or LC_{50} values. The positive and negative values are plotted along a vertical line that represents the mean response of all the cell lines in the panel to the test agent. Positive values project to the right of the vertical line and represent cellular sensitivities to the test agent that exceed the mean. Negative values project to the left and represent cell line sensitivities to the test agent that are less than the average value.

The positive and negative values, called deltas, are generated from the GI_{50} data (or TGI or LC_{50} data) by a three-step calculation. The GI_{50} value for each cell line tested against a test compound is converted to its $\log_{10}$ GI_{50} value. These $\log_{10}$ GI_{50} values are averaged. Each $\log_{10}$ GI_{50} value is subtracted from the average to create the delta. Thus, a bar projecting 3 units to the right denotes that the GI_{50} (or TGI or LC_{50}) for that cell line occurs at a concentration 1000 times less than the average concentration required for all the cell lines used in the experiment.

Correlation analysis [18]

COMPARE analyses were run to find similarity of *in vitro* cell growth pattern of NSC 726577 and NSC 726578 to the *in vitro* cell growth pattern of compounds from two distinct databases (Synthetics database and Molecular Targets database).

Average difference method

The first step in calculating this index of similarity is to take the difference between paired Δs. Each pair consists of the Δ value from the seed for a particular cell line and the Δ value of a database compound for the same cell line. An average of these differences, by compound, is computed for each compound. The compounds are sorted by their average difference. The compound with the smallest average difference is the most similar to the seed compound. A description of the COMPARE methodology can be found on the DTP website at http://dtp.nci.nih.-gov/docs/compare/compare.html.

Correlation coefficient method

A pairwise Pearson's correlation coefficient (PCC) with the seed is calculated for each compound in the database. Those compounds with the highest correlation coefficient are most similar to the seed. The higher the PCC is, the more similar are the patterns of growth inhibition. However, high correlations can be observed for compounds for which variability between cell lines is not high and therefore one must examine the data underlying COMPARE results.

Results and discussion

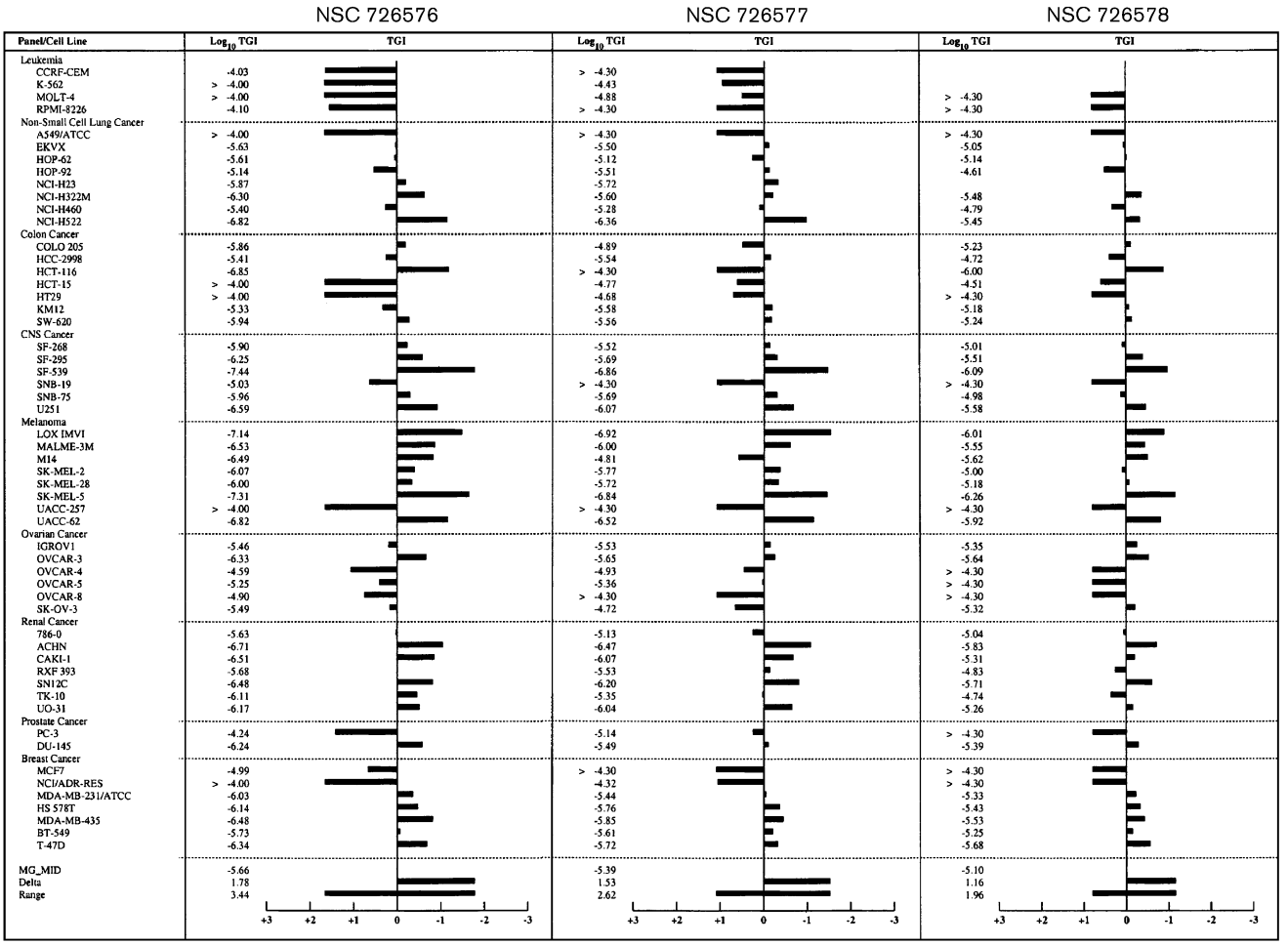
The growth inhibitory activity and cytotoxicity of NSC 726576, NSC 726577 and NSC 726578 towards the NCI panel of 60 human tumor cell lines was assessed by the SRB assay at 48 h of drug exposure. The dose–responses curves (not reported here) showed that the three indolocarbazole derivatives possess different selectivity profiles. Their cytostatic effects were determined by the analysis of the corresponding mean graphs (Fig. 2). The results of the mean graph analysis showed that, on leukemia cells, the three rebeccamycin derivatives exhibited no cytostatic significant effect, whereas, in previous studies, the three compounds were found to have IC_{50} values about 0.1 μM on K-562 leukemia cells [15]. The discrepancy observed is probably due to the different growth conditions used for the two assays. For the other cells, two categories of cancer cells were distinguished: the first one for which the indolocarbazole compound has a cytostatic effect on almost all cell lines and the second one for which a cytostatic effect was observed on only a few cell lines.

On all the melanoma cells tested, except UACC-257, NSC 726576 induced a cytostatic effect with a TGI in the 10^{-6} to 10^{-7} M range. On all the renal cancer cells tested, except 786-0 cells, the TGI was in the 10^{-5} to 10^{-6} M range. On all the breast cancer cells lines tested, except NCI/ADR-RES and MCF7, the TGI was in the 10^{-5} to 10^{-6} M range. Moreover, NSC 726576 presented a cytostatic effect toward all the CNS cancer lines tested, except SNB-19, with a TGI in the 10^{-5} to 10^{-6} M range.

In the second category, a TGI in the 10^{-6} M range was only observed on HCT-116 (colon cancer cells), OVCAR-3 (ovarian cancer cells), DU-145 (prostate cancer cells), NCI-H522 and NCI-H322 M (non-small cell lung cancer).

Compared to NSC 726576, NSC 726577 showed a similar profile on the breast cancer, the prostate cancer and the CNS cancer cells tested. In contrast, NSC 726577 showed no cytostatic effect on TK-10 (renal cancer cells), COLO 205 and HCT-116 (colon cancer cells), and M-14 (melanoma cells), whereas it was active on HOP-92 and EKVX (non-small cell lung cancer), IGROV1 (ovarian cancer cells), and KM12 and HCC-2998 (colon cancer cells) with TGIs in the 10^{-5} M range. Compared to NSC 726576 and NSC 726577, NSC 726578 exhibited similar cytostatic effects on the breast cancer and the prostate cancer cell lines tested. The cytostatic profile of NSC 726578 was almost the same as that of NSC 726577 for renal and ovarian cancer except that it was inactive on RFX 393 (renal cancer cells) and SK-OV-3 (ovarian cancer cells) with TGIs in the 10^{-5} M range. The cytostatic profiles of NSC 726578 and NSC 726576 were similar for CNS cancer cell lines and for the non-small cell lung cancer cell lines tested. In contrast to that observed for NSC 726576, NSC 726578 was inactive on SK-MEL-2 (melanoma cells). The NSC 726578 cytostatic effects on

Fig. 2



TGI mean graphs for the three compounds tested. TGI (M) is the drug concentration resulting in a total growth inhibition.

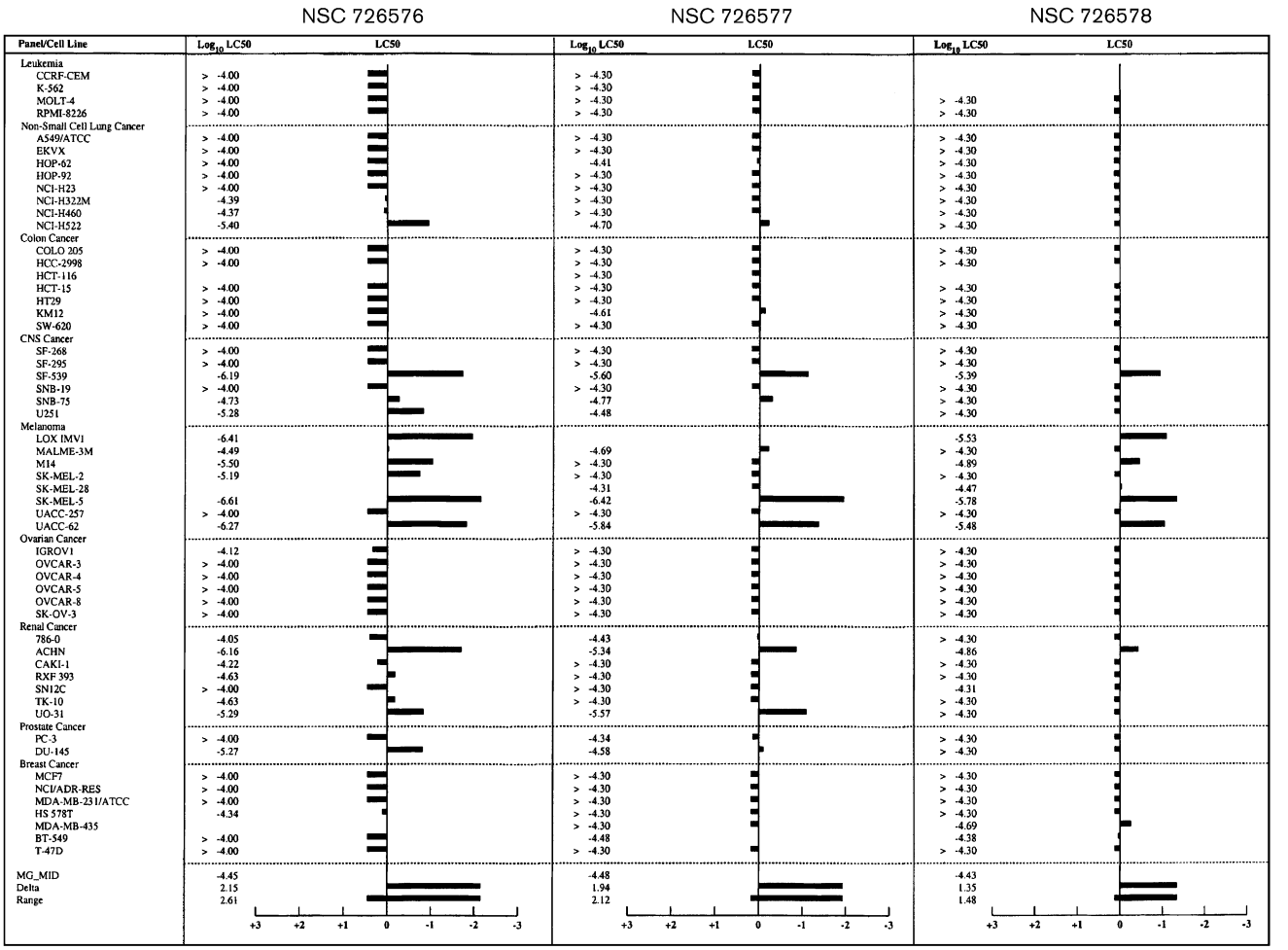
the colon cancer cell lines tested were different from those of the other indolocarbazoles, this compound was only active on HCT-116, KM12 and SW-620 with TGIs in the 10^{-5} M range.

The mean graphs of LC_{50} values (Fig. 3) describe the cytotoxic activities of these three compounds. NSC 726576 was the most potent—it showed cytotoxicity on 14 cell lines in the 60 tested. The most sensitive cell lines were the melanoma cells with $\log_{10}$ LC_{50} in the -5.19 to -6.61 M range and the renal cells with $\log_{10}$ LC_{50} in the -4.63 to -6.16 M range. NSC 726577 was less potent—it exhibited cytotoxicity on only 10 cell lines in the 60 tested. The most sensitive cell lines toward this compound were the melanoma cells with $\log_{10}$ LC_{50} in the -4.69 to -6.42 M range. NSC 726578 inhibited only eight cell lines in the 60 tested. The most sensitive cell lines are once more the melanoma cells with $\log_{10}$ LC_{50} in the -4.89 to -5.78 M range. NSC 726576 was the most potent compound in terms of cytotoxicity, but it was not selective. NSC 726577 and NSC 726578 are less potent, but they showed a better selectivity; both compounds for melanoma cell lines, and NSC 726577 for some CNS cancer and the renal cancer cell lines tested.

To understand the differences between the cytostatic effects and the cytotoxicity profiles of this three rebeccamycin analogs, COMPARE analyses were ran with NSC 726576 (dechlorinated rebeccamycin), NSC 726577 and NSC 726578 to find any similarity in their *in vitro* cell growth pattern and those of compounds from the NCI's Standard Agents database, the NCI's Synthetics database and NCI's Molecular Targets database.

Against the Standard Agents database, NSC 726577 shows primarily correlations with DNA-binding agents (e.g. deoxydoxorubicin and daunomycin), but also with flavopiridol (PCC of 0.44 at GI_{50}). NSC 726578 showed

Fig. 3



LC_{50} mean graphs for the three compounds tested. LC_{50} (M) is the concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning.

similar correlations with DNA-binding agents, but did not show the correlation with flavopiridol. Flavopiridol is a cyclin-dependent kinase inhibitor with IC_{50} values of 20–60 nM on CDK1/cyclin B, CDK4/cyclin D and CDK6/cyclin D. These results are in agreement with the stronger inhibitory activity of NSC 726577 toward the kinases tested (Table 1).

Table 1 Inhibitory activities toward three kinases (IC_{50} μM)

Compound	CDK1/cyclin B	CDK5/P25	GSK-3
Rebeccamycin	>5	>5	0.7
NSC 726576	>10	>5	0.7
NSC 726577	<5	<5	0.5
NSC 726578	<5	>5	2

$IC_{50} < 5$ means $1 < IC_{50} < 5$.

Unfortunately comparison analysis made with NCI Synthetics database showed for NSC 726576 correlations primarily with Topo I inhibitors (camptothecins and rebeccamycin), and secondarily with a protein synthesis inhibitor (verrucarin A), Topo II inhibitors (daunorubicins), a DNA-binding agent (amiguanidine) and a tubulin-binding drug (combrestatin). For NSC 726577 and NSC 726578, good correlations were observed with DNA-binding and Topo agents. For NSC 726577, four compounds known for interacting with DNA and Topos (NSC 681641, chartreusin, daunorubicin and U 25795) have been found in the 25 best PCC (0.525–0.675), whereas for NSC 726578 five compounds (U 25795, daunorubicin, actinomycin, NSC 693574 and NSC 639659) were found in the 25 best results (PCC range 0.565–0.758).

The TGI and LC_{50} correlations between our compounds and a rebeccamycin derivative NSC 655649 (BMS 181176, Fig. 1), which is known to inhibit Topo II and which is currently undergoing clinical trials, are shown in

Table 2 PCC values of TGI correlation analysis

Compound	NSC 655649	NSC 726576	NSC 726577	NSC 726578
NSC 655649		0.28	0.19	0.41
NSC 726576	0.28		0.9	0.71
NSC 726577	0.19	0.9		0.61
NSC 726578	0.41	0.71	0.61	

Table 3 PCC values of LC_{50} correlation analysis

Compound	NSC 655649	NSC 726576	NSC 726577	NSC 726578
NSC 655649		0.13	0.11	0.32
NSC 726576	0.13		0.82	0.56
NSC 726577	0.11	0.82		0.62
NSC 726578	0.32	0.56	0.62	

Tables 2 and 3. Good correlations were observed between NSC 726576, NSC 726577 and NSC 726578, but no significant correlations were observed between these compounds and NSC 655649, suggesting that Topo I is more the target rather than Topo II for these three compounds.

Conclusion

In conclusion, this study has shown that the rebeccamycin derivatives, NSC 726576, NSC 726577 and NSC 726578, have different cytostatic and cytotoxic effects. The three compounds are active and selective toward some of the tumor cell lines tested. Binding to DNA and Topo I inhibition seem to be the major mechanisms of action of the three compounds. Moreover, kinases are also targets for NSC 726577 as indicated by the correlation with flavopiridol.

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