# DIVERSE EFFECTS OF INDOLOCARBAZOLE COMPOUNDS ON THE CELL CYCLE PROGRESSION OF *ras*-TRANSFORMED RAT FIBROBLAST CELLS

SHIRO AKINAGA*, KAYO NOMURA, KATSUSHIGE GOMI and MASAMI OKABE

Pharmaceutical Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., Shimotogari 1188, Nagaizumi-cho, Sunto-gun, Shizuoka 411, Japan

(Received for publication June 7, 1993)

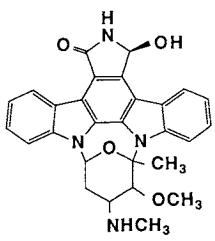
Indolocarbazole compounds have been reported to exert antitumor activity against human and murine tumor cell lines *in vitro* and/or *in vivo*[1~13]. These compounds are thought to be divided into two major groups, *i.e.* protein kinase inhibitors and topoisomerase inhibitors[7]. The former includes natural compounds such as staurosporine[1], K-252a[2] and UCN-01[3~5] as well as chemically synthesized derivatives of the natural compounds[2,6,11]. The latter includes natural compounds such as rebeccamycin[8], AT2433[9] and several derivatives of the natural compounds which did not exert the apparent inhibitory activities against protein kinases[7,10~12].

Recent studies showed that staurosporine and K-252a exhibited a unique effect on the cell cycle distribution of mammalian cells[13~17]. At lower concentrations, for example, they induced a G1 (or G1/S) phase accumulation in the cell cycle in a reversible manner, however, at higher concentrations, they exhibited a block of the cell cycle at G2M phase[13~17]. In addition, staurosporine[16] and/or K-252a[15] induced a higher DNA ploidy in some types of cells. However, at least to our knowledge, there have been few studies comparing the cell cycle effects of indolocarbazole compounds in relation to their different biological effects.

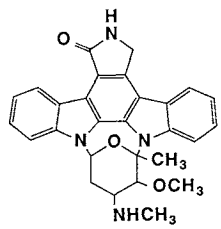
In this study, we compared the effects of non-selective protein kinase inhibitors (staurosporine[1,7] and K-252a[2,7]), protein kinase C (PKC) selective inhibitors (UCN-01[3~5] and CGP 41 251[6]), and topoisomerase I inhibitors (the derivatives of K-252a, KT6124, KT6006 and KT6528[12]) on the cell cycle distribution of *ras*-transformed rat 3Y1 cells, HR-3Y1[15,18]. This cell line was selected for this study because 1) it was reported that K-252a had induced DNA higher ploidy in this cell line more effectively than in other cell lines[15] and 2) it was suggested that *ras*-activated tumor cell lines might be a target for PKC inhibitors such as UCN-01[5]. The chemical structures of all the compounds used in this study are shown in Fig. 1.

The inhibitory activities of these indolocarbazole

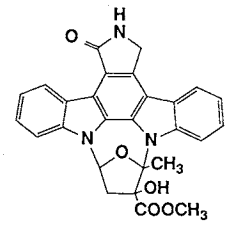
Fig. 1. Structures of indolocarbazole compounds examined in this study.



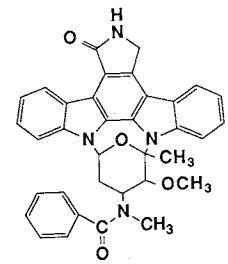
UCN-01



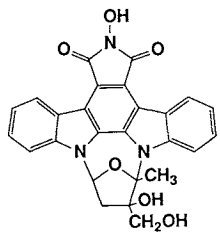
Staurosporine



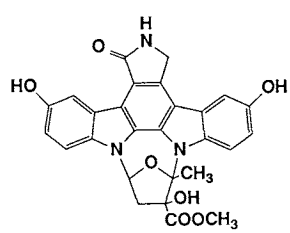
K-252a



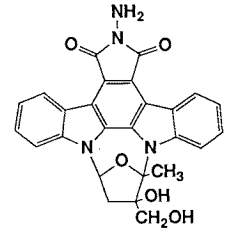
CGP 41 251



KT6528



KT6006



KT6124

Table 1. Biological properties of indolocarbazole compounds examined in this study.

| Compound | $IC_{50}$ ($\mu$M) | | Topoisomerase I inhibition | Reference |
|---|---|---|---|---|
| | PKC[a] | PKA[b] | | |
| Staurosporine | 0.0027 | 0.0054 | No | 1, 12 |
| K-252a | 0.020 | 0.020 | No | 2, 12 |
| UCN-01 | 0.0041 | 0.042 | No | 3, 12 |
| CGP 41 251 | 0.050 | 2.4 | No | 6, this study |
| KT6124 | 0.47 | 0.47 | Yes | 11, 12, this study |
| KT6528 | 3.84 | 0.96 | Yes | 12 |
| KT6006 | 0.040 | 0.040 | Yes | 12 |

[a] PKC; protein kinase C.
[b] PKA; protein kinase A.

Table 2. Effect of indolocarbazole compounds on the cell-cycle distribution of HR-3Y1 cells by 24 hours exposure.

| Drug | Concentration ($\mu$M) | Growth inhibition (%) | Cell cycle distribution (%) | | | |
|---|---|---|---|---|---|---|
| | | | G1 | S | G2M | >G2M |
| None | — | — | 40.4 | 48.4 | 11.2 | — |
| Staurosporine | 0.25 | 53 | 5.0 | 8.7 | 65.1 | 21.1 |
| K-252a | 2.3 | 54 | 2.3 | 6.1 | 63.7 | 27.9 |
| UCN-01 | 1.2 | 53 | 82.0 | 10.6 | 7.4 | — |
| CGP 41 251 | 10 | 54 | 3.2 | 3.0 | 28.1 | 65.7 |
| KT6124 | 2.9 | 55 | 35.9 | 52.2 | 11.9 | — |
| KT6528 | 1.0 | 60 | 15.5 | 69.2 | 15.2 | — |
| KT6006 | 6.3 | 60 | 15.7 | 38.6 | 26.7 | 19.0 |

Exponentially growing HR-3Y1 cells were treated with the drugs at the indicated concentrations for 24 hours, and the cell cycle distribution was determined from DNA histograms in Fig. 1 by the MULTICYCLE program. Cell growth inhibition of each drug was determined by MTT method.

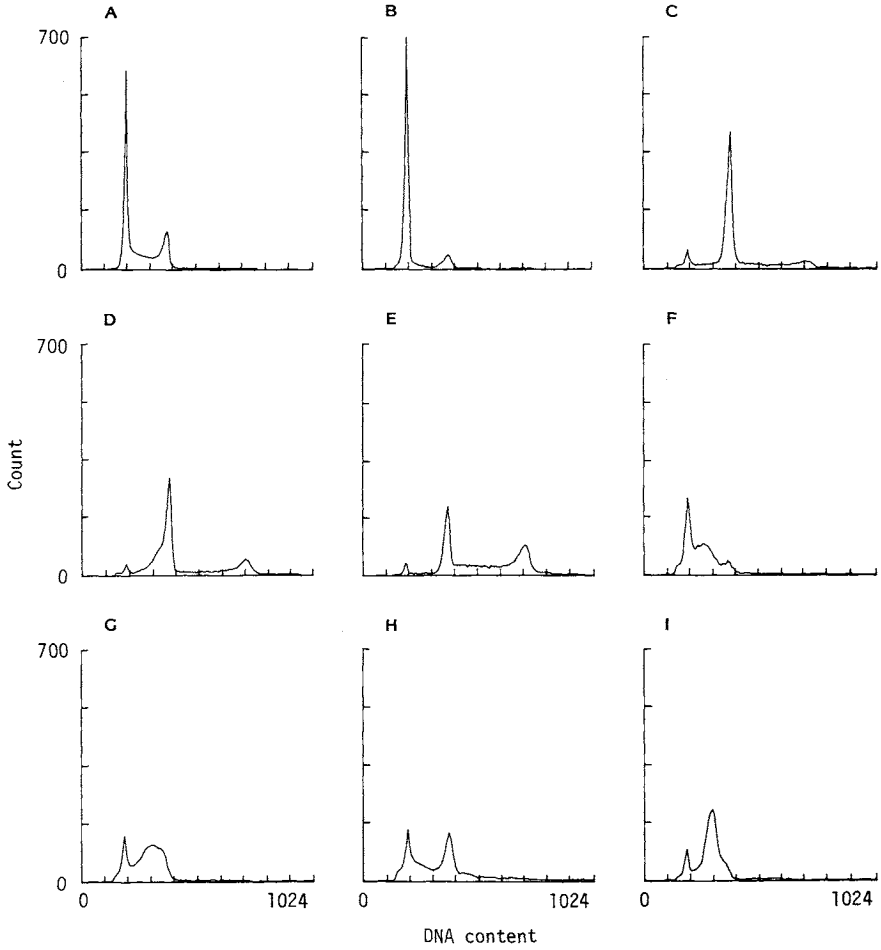
compounds against PKC, protein kinase A (PKA) and topoisomerase I are summarized in Table 1. To compare the cell cycle effects of all the indolocarbazole compounds used in this study under the same condition, the flow cytometric analysis was performed after 24 hours exposure of the cells to the drugs under the cytostatic conditions as shown in Table 2.

Non-selective protein kinase inhibitors, *i.e.* staurosporine and K-252a induced a typical G2/M phase accumulation and also higher DNA ploidy (Figs. 2C, 2D and Table 2) as previously reported[15,16]. In contrast to this effect, UCN-01, a selective inhibitor of PKC, exerted a preferential G1 phase block of the cell cycle without any higher DNA ploidy (Fig. 2B and Table 2). However, another type of selective inhibitor of PKC, CGP 41 251 exerted the G2M phase block with higher DNA ploidy (Fig. 2E and Table 2) in a similar manner to staurosporine and K-252a. The appearance of giant cells with polyploidy 24 hours after the treatment with CGP 41 251 as well as staurosporine or K-252a was also confirmed by microscopic observation (data not shown).

On the contrary, KT6124, KT6528 and KT6006, which were the inhibitors of topoisomerase I (Table 1), exhibited quite different effects on the cell cycle (Figs. 2F, 2G, 2H and Table 2). KT6124 and KT6528 showed apparent S phase delay of the cell cycle progression (Figs. 2F and 2G) which resembles the effect of camptothecin, a well-known topoisomerase I inhibitor[12] (Fig. 2I and Table 2). However, KT6006 induced not only the S phase delay but also G2M phase block with a small population of higher DNA ploidy as shown in Fig. 2H and Table 2.

These results show that seven indolocarbazole compounds with minor stuructural differences were found to cause quite different effects on the cell cycle progression of *ras*-transformed rat fibroblast cells (HR-3Y1). The effects of these compounds on the cell cycle distribution are classified into the following four groups. The first group consists of staurosporine, K-252a and CGP 41 251 that cause the G2M phase block and the higher DNA ploidy, which

Fig. 2. DNA histograms of HR-3Y1 cells treated with indolocarbazole compounds and camptothecin.




HR-3Y1 cells ($3 \times 10^5$ cells/20 ml/dish) were cultured on day 0, and treated with (A) culture medium alone, (B) UCN-01 (1.2 $\mu$M), (C) staurosporine (0.25 $\mu$M), (D) K-252a (2.3 $\mu$M), (E) CGP 41 251 (10 $\mu$M), (F) KT6124 (2.9 $\mu$M), (G) KT6528 (1.0 $\mu$M), (H) KT6006 (6.3 $\mu$M) and (I) camptothecin (0.28 $\mu$M) on day 1. The cells were fixed for flow cytometric analysis 24 hours after the addition of the drugs. DNA histograms were obtained by a flow cytometry.

was reported previously for staurosporine[16] and K-252a[15]. These effects were thought to be mediated through, at least in part, the inhibition of cdc2 kinase[15,19] which was supposed to play a central role in the transition of the G2 to M phase in the cell cycle progression[20].

The second type represented by UCN-01 is the preferential induction of the G1 phase block (Fig. 2B), although this compound selectively inhibits PKC as does CGP 41 251. A similar pattern of G1 phase accumulation was also detected when the cells were treated with lower concentrations of staurosporine and K-252a[13~16] (data not shown), suggesting that the inhibition of serine/threonine kinases including PKC might play an important role in the induction of the G1 phase block. In addition, the inhibition of cdk2 kinase might be important for the induction of the G1 phase accumulation by these compounds, because cdk2 is supposed to play an important role in the G1 to S phase transition in cell cycle progression[20]. A possible inhibition of cdk2 kinase by these compounds remains to be determined.

The third type is the induction of S phase delay represented by KT6124 and KT6528 (Figs. 2F and 2G). This effect was never detected in the cells treated with staurosporine, K-252a, CGP 41 251 and UCN-01. The pattern of cell cycle effect is similar

to that of camptothecin (Fig. 2I and ref 21). In addition, KT6124 was shown to exert DNA single strand breaks[11] which was not induced by UCN-01 and staurosporine[5] in the other intact cells, suggesting that KT6124 could act as an inhibitor of topoisomerase I in intact cells.

The last type represented by KT6006 exerted a somewhat different effect on the cell cycle (Fig. 2H) from the other compounds. This compound exhibited both S phase delay and G2M block with higher DNA ploidy. We have previously shown that KT6006 but not KT6124 and KT6528 inhibits the action of PMA in intact cells[11] (unpublished results) as is the case for staurosporine[5], K-252a[11], UCN-01[5] and CGP 41 251 (unpublished results). Taken together, the effect of KT6006 on the cell cycle distribution may be mediated through both the inhibition of topoisomerase I and protein kinases.

In conclusion, the indolocarbazole compounds were found to exhibit diverse effects on the cell cycle progression of *ras*-transformed rat fibroblast cells, HR-3Y1, suggesting that there are several modes of action of these compounds on the cells. Although further analysis of the effect of each indolocarbazole compound on the cell cycle remains to be done, cell cycle analysis using HR-3Y1 cells may be helpful for the characterization and/or prescreening of novel compounds of this class.

Acknowledgments

The aurhors thank Drs. HIROFUMI NAKANO and YOSHINORI YAMASHITA for the determination of topoisomerase I activity. We also thank Drs. TATSUYA TAMAOKI and TADASHI HIRATA for the critical review of the manuscript and the encourangement during the studies. We are grateful to Miss SHINOBU KOYAMA and Miss YUKA WATANABE for their excellent technical assistance.

## References

1) TAMAOKI, T.; H. NOMOTO, I. TAKAHASHI, Y. KATO, M. MORIMOTO & F. TOMITA: Staurosporine, a potent inhibitor of phospholipid/$Ca^{2+}$ dependent protein kinase. Biochem. Biophys. Res. Commun. 135: 397~402, 1986
2) KASE, H.; K. IWAHASHI & Y. MATSUDA: K-252a, a potent inhibitor of protein kinase C from microbial origin. J. Antibiotics 39: 1059~1065, 1986
3) TAKAHASHI, I.; E. KOBAYASHI, K. ASANO, M. YOSHIDA & H. NAKANO: UCN-01, a selective inhibitor of protein kinase C from *Streptomyces*. J. Antibiotics 40: 1782~1784, 1987
4) TAKAHASHI, I.; Y. SAITO, M. YOSHIDA, H. SANO, H. NAKANO, M. MORIMOTO & T. TAMAOKI: UCN-01 and UCN-02, new selective inhibitors of protein kinase C. II. Purification, physico-chemical properties, structural determinations and biological activities. J. Antibiotics 42: 571~576, 1989
5) AKINAGA, S.; K. GOMI, M. MORIMOTO, T. TAMAOKI & M. OKABE: Antitumor activity of UCN-01, a selective inhibitor of protein kinase C, in murine and human tumor models. Cancer Res. 51: 4888~4892, 1991
6) MEYER, T. T.; U. REGENASS, D. FABBRO, E. ALTERI, J. ROSEL, M. MULLER, G. CARAVATTI & A MATLER: A detivative of staurosporine (CGP41 251) shows selectivity for protein kinase C inhibition and *in vitro* anti-proliferative as well as *in vivo* and·tumor activity. Int. J. Cancer 43: 851~856, 1989
7) TAMAOKI, T. & H. NAKANO: Potent and specific inhibitors of protein kinase C of microbial origin. Biotechnology 8: 732~735, 1990
8) BUSH, J. A.; B. H. LONG, J. J. CATINO, W. T. BRADNER & K. TOMITA: Production and biological activity of rebeccamycin, a novel antitumor agent. J. Antibiotics 40: 668~678, 1987
9) MATSON, J. A.; C. CLARIDGE, J. A. BUSH, J. TITUS, W. T. BRADNER, T. W. DOYLE, A. C. HORAN & M. PATEL: AT2433-A1, AT2433-A2, AT2433-B1, and AT2433-B2 novel antitumor antibiotics compounds produced by *Actinomadura melliaura*. Taxonomy, fermentation, isolation and biological properties. J. Antibiotics 42: 1547~1555, 1989
10) TANAKA, S.; M. OHKUBO, K. KOJIRI, H. SUDA, A. YAMADA & D. UEMURA: A new indolopyrrolocarbazole antitumor substance, ED-110, a derivative of BE-13793C. J. Antibiotics 45: 1797~1798, 1992
11) AKINAGA, S.; T. ASHIZAWA, K. GOMI, H. OHNO, M. MORIMOTO, C. MURAKATA & M. OKABE: Antitumor effect of KT6124, a novel derivative of protein kinase inhibitor K-252a, and its mechanism of action. Cancer Chemother. Pharmacol. 29: 266~272, 1992
12) YAMASHITA, Y.; N. FUJII, C. MURAKATA, T. ASHIZAWA, M. OKABE & H. NAKANO: Induction of mammalian DNA topoisomerase I mediated DNA clavage by antitumor indolocarbazole derivatives. Biochemistry 31: 12069~12075, 1992
13) ABE, K.; M. YOSHIDA, T. USUI, S. HORIUCHI & T. BEPPU: Highly synchronous culture of fibroblasts from G2 block caused by staurosporine, a potent inhibitor of protein kinases. Exp. Cell Res. 192: 122~127, 1991
14) CRISSMAN, H. A.; D. M. GADBOIS, R. A. TOBEY & E. BRADBURY: Transformed mammalian cells are deficient in kinase-mediated control of progression through the G1 phase of the cell cycle. Proc. Natl. Acad. Sci. U.S.A. 88: 7580~7584, 1991
15) USUI, T.; M. YOSHIDA, K. ABE, H. OSADA, K. ISONO & T. BEPPU: Uncoupled cell cycle without mitosis induced by a protein kinase inhibitor, K-252a. J. Cell Biol. 115: 1275~1282, 1991

16) BRUNO, S.; B. ARDELT, J. S. SKIERSKI, F. TRAGANOS & Z. DARZYNKIEWICZ: Different effects of staurosporine, and inhibitor of protein kinases, on the cell cycle and chromatin structure of normal and leukemic lymphocytes. Cancer Res. 52: 470～473, 1992

17) GADBOIS, D. M.; H. A. CRISSMAN, R. A. TOBEY & E. M. BRADBURY: Multiple kinase arrest points in the G1 phase of nontransformed mammalian cells are absent in transformed cells. Proc. Natl. Acad. Sci. U.S.A. 89: 8626～8630, 1992

18) OHNO, K.; A. OKUDA, M. OHTSU & G. KIMURA: Genetic analysis of control of proliferation in fibroblastic cells in culture. I. Isolation and characterization of mutants temperature-sensitive for ploriferation of survival of untransformed diploid rat cell line 3Y1. Somat. Cell Mol. Genet. 10: 17～28, 1984

19) GADBOIS, D. M.; J. R. HAMAGUCHI, R. A. SWANK & E. M. BRADBURY: Staurosporine is a potent inhibitor of $p34^{cdc2}$ and $p34^{cdc2}$-like kinases. Biochem. Biophys. Res. Commun. 184: 80～85, 1992

20) NORBURY, C. & P. NURSE: Animal cell cycles and their control. Annu. Rev. Biochem. 61: 441～470, 1992

21) BINO, G. D.; J. S. SKIERSKI & Z. DARZYNKIEWICZ: Diverse effects of camptothecin, an inhibitor of topoisomerase I, on the cell cycle of lymphocytic (L1210, MOLT-4) and myelogenous (HL-60, KG-1) leukemia, Cancer Res. 50: 5746～5750, 1990