Eur J Cancer Clin Oncol, Vol. 24, No. 8, pp. 1385–1389, 1988.
Printed in Great Britain

0277-5379/88$3.00+0.00

Special Article

Membrane Targets in Cancer Chemotherapy

A Report of a One Day Symposium of the EORTC-PAM Group Meeting Held at the Centre Antoine Lacassagne, Nice, France, 4 December 1987

J.A. HICKMAN* for the EORTC Phamacokinetics and Metabolism Group

**Cancer Research Campaign Experimental Chemotherapy Group, Pharmaceutical Sciences Institute, Aston University, Aston Triangle, Birmingham B4 7ET, U.K.*

ANTICANCER DRUG discovery is often a perilously serendipitous pursuit. Nevertheless, many successful antitumour drugs have been designed with an understanding of the biochemistry of replication acting as a framework for the medicinal chemist and molecular pharmacologist. One of the current problems of antitumour drug discovery is the question of whether the particular framework which has served for the past 40 years was the most appropriate. Cancer is primarily a disease of the aberrant *control* of cellular differentiation and proliferation: the 'mechanics' of the replication of DNA are not at the heart of the malignant phenotype. Where then might the aspiring drug discoverer turn his thoughts, and find a biochemical framework that may be more appropriate? One such target might be the cell membrane, where elements of the control of cell differentiation and proliferation are expressed [1, 2], and where the products are found of a number of oncogenes, which appear to subvert the control of differentiation and proliferation [3].

At a recent meeting of the EORTC-PAM group, some of the cell membrane physiology and biochemistry associated with the control of the mitogenic response was reviewed, accounts of the effects of some current antitumour drugs on these processes were considered and the symposium closed with presentations on novel drugs which are considered to exert their antitumour effects via membrane targets.

Accepted 5 April 1988.

Wouter Moolenaar (Hubrecht Laboratory, Utrecht), in an opening review, outlined the early events which result from the mitogenic stimulation of A431 carcinoma or N1E-115 neuroblastoma cells by epidermal growth factor (EGF) and by mitogenic neurohormones, such as histamine and bradykinin. These early events include changes in the phosphorylation of the tyrosine residues of proteins, such as receptor autophosphorylation, the phosphorylation of calpactin and the breakdown of inositol phospholipids to inositol phosphates and diacylglycerol, the former which releases intracellular stores of calcium whilst the latter activates protein kinase C. Activation of the Na^+/H^+ antiporter, possibly via a stimulation of protein kinase C, rapidly changes intracellular pH [4]. These changes take place within milliseconds to minutes, although epidermal growth factor must be present for at least 14 h in order to stimulate cell division. The central question of which of the early events are necessary for mitogenic stimulation was addressed by Dr Moolenaar. In particular, he addressed the relevance of those changes which may impinge upon the rapid stimulation of the transcription of genes, such as *fos*, which is considered to pay a major role in cellular activation [5]. Elements of commonality were sought between the responses to EGF and histamine. For example, although both mitogens stimulated the formation of inositol phosphates, there were quantitative and qualitative differences in their breakdown, with EGF promoting a slower and diminished inositol lipid breakdown by a route presumably different from that of histamine. However, the stimulation

of *fos* transcription was similar for both ligands. This stimulation appeared to be Ca^{2+}-dependent in the case of the neurohormones, but Ca^{2+}-independent in the case of EGF. That separate signalling pathways exist, which converge to activate gene transcription, suggests that pharmacological opportunities may exist for the selective inhibition of some of these events, perhaps under conditions when the activity of a growth factor receptor is expressed as the product of an oncogene, as is the case of the *erb-B* oncogene, which is homologous to the internal domain of the EGF receptor.

Sonia Paris (CNRS, Nice) focussed on two of the early events observed at the cell membrane after the mitogenic stimulation of Chinese hamster lung fibroblasts [6]: the alkalinization of intracellular pH, modulated by the Na^+/H^+ antiport when the cells were in bicarbonate media, and the rapid stimulation of the electroneutral $Na^+K^+Cl^-$ cotransporter, considered to play a role in the volume control of some cells. Thrombin was considered to stimulate mitogenesis via the activation of inositol lipid metabolism and, since the activity of thrombin was inhibited by pertussis toxin, it was presumed that lipid breakdown is activated via a G-protein, coupling the receptor to phospholipase C. Recent dogma has maintained that the breakdown of inositol lipid to diacylglycerol (DAG) activates the Na^+/H^+ antiport through activation of protein kinase C, for which DAG is the natural activating ligand. Pertussis toxin only partially inhibited the alkalinization of the cell, however, and furthermore thrombin was reported by Dr Paris and her colleagues to stimulate a pH change even after the down regulation of protein kinase C by pretreatment of the cells with the phorbol ester TPA. It was suggested that other pathways of activation exist to modulate these early events, an idea perhaps supported by the strong synergy between different growth factors, acting via or independently of inositol lipid breakdown in this cell line [7]. Whatever the route of activation of cellular alkalinization, Dr Paris suggested that the change in pH was essential to cell activation, possibly by modifying the pH optima of key enzymes required to set mitogenesis in motion. Less clear was the role of the $Na^+K^+Cl^-$ cotransporter, since bumetanide, a specific inhibitor of its activity, does not inhibit mitogenesis [8]. One possible explanation for the lack of the effect of bumetanide on mitosis is that the cell may regulate its volume by alternative mechanisms, by-passing the $Na^+K^+Cl^-$ cotransporter.

The complexity of the signalling systems which initiate mitogenesis, reviewed by Drs Moolenaar and Paris, appears to be daunting; however, because there is in-built complexity, for example multiple modes of the activation of gene expression (e.g. of *fos*), this may, paradoxically, be a positive facet with regard to the potential for selective pharmacological inhibition. More daunting would be the prospect of attempting to selectively inhibit a limiting number of signalling pathways which were highly conserved in all cells, in addition to those with proliferative potential. Recent studies suggest that some of the key enzymes involved in the mitogenic cascade, such as protein kinase C, exist in isoenzymic forms [9], which implies a potential for selective pharmacological intervention, whilst selective inhibitors of tyrosine kinases and protein kinase C have recently been reported [10].

Dr Anne Kinsella (Paterson Laboratories, Manchester) reviewed the pleiotropic effects of the tumour promoting phorbol esters, which bind to and activate the ubiquitous calcium and phospholipid-sensitive enzyme protein kinase C. According to the cell type, phorbol esters, such as TPA, are mitogenic or growth inhibitory, and it has been suggested by Varshavsky [11] that TPA may behave as a non-specific 'firone', bringing about the over-replication and thus amplification of certain genes, particularly dihydrofolate reductase. Dr Kinsella provided evidence that the resistance of TPA-treated murine 3T3 cells to methotrexate was not due to gene amplification [12] but instead was brought about by modulation of cell growth, resulting in the accumulation of cells in the G_1 phase of the cell cycle, presumably allowing them to escape the S-phase specific effects of the antifolate drug [13].

Tom Tritton (Burlington, Vermont) initiated the pharmacological business of the meeting by provocatively proposing that the cytotoxic drug Adriamycin (Doxorubicin) may, under certain conditions, act as a stimulus to cell growth [14]. Evidence was presented of the 50% stimulation of murine 3T3 cells by 1 nM of the drug, and of its augmentation of the growth stimulus provided by platelet derived growth factor and EGF. Previous work from his laboratory had shown the up-regulation of EGF receptors in Adriamycin-treated HeLa cells [15]. Dr Tritton proposed that Adriamycin may share elements of the activity of the growth factors which are expressed at the cell surface but that at high concentrations these effects may, in an undefined manner, interfere with the normal control of proliferation. Adriamycin was shown to rapidly change tyrosine phosphorylation and to increase the turnover of inositol lipids, although, puzzlingly, the expected rise in intracellular calcium which inevitably follows the release of inositol trisphosphate, accompanied by diacylglycerol, was absent. Paradoxically, others have reported that Adriamycin inhibits inositol lipid breakdown in the red cell, where it controls cell shape; it is currently being suggested that

discrete pools of the inositol lipids exist in the cell membrane, some of which may not be involved in cell signalling, and thus these contradictory results on the effects of Adriamycin on inositol lipid metabolism may be reconcilable [16]. Tom Tritton considered that the effects of this amphipathic aminoglycoside antibiotic were the result of physical changes in membrane structure, brought about by the molecule mechanically disrupting the architecture of the bilayer. The potential consequences of these activities—and in particular the activation of protein kinase C by the released diacylglycerol—were the subsequent activation of the DNA-nicking and reannealing enzyme topoisomerase II. If this were the case, then a scenario of membrane and nuclear damage might be envisaged, in which the activation by diacylglycerol of topoisomerase II would be followed by the accumulation of protein-associated double strand breaks in DNA, as topoisomerase II was stabilized in the form attached to cleaved DNA, as has been suggested by Ross and others [17].

Equally provocative were the accounts of Dr Hans Grunicke (Innsbruck) of the cell surface effects of the archetypal DNA-reactive drugs, the alkylating agents. Dr Grunicke has previously suggested that chlorambucil linked to large polymeric beads is cytotoxic without damaging DNA [18]. Together with others, Grunicke's group has shown the alkylating agents inhibit ion flux, and in particular the $Na^+K^+Cl^-$ cotransporter of murine tumour cells [19]. Interference with events associated with the mitogenic cascade suggested that coadministration of agents which have inhibitory activities at proximal points in the cascade may potentiate toxicity. Data was presented which suggested that the inhibitor of cyclic AMP-independent protein kinases, the flavone quercetin, had a synergistic effect on the toxicity of the alkylating agents and *cis*-platinum, without increasing DNA damage, as measured by the number of DNA interstrand crosslinks. Similar synergisms were observed with staurosporine and tamoxifen, both agents which inhibit protein kinase C [10].

The inhibition of proliferation and the imposition of cytotoxicity are the traditional end-points of chemotherapy. Whether this is appropriate to a disease in which proliferation control has been subverted by a block in the path of cellular differentiation is questionable. Recently, the potential for a chemotherapy which is not cytotoxic has been proposed: instead of killing tumour cells, it aims to remove the block in cellular differentiation, promoting the terminal differentiation or maturation of tumours [21]. The dependency of the proliferative potential of a cell on its differentiated status not surprisingly means that the controls for differentiation and proliferation are linked. Changes in the flux of ions and protein phosphorylation, mediated at the level of the membrane, as leukaemic cells were induced to undergo terminal differentiation were the subject of the presentation by Dr Jean-Pierre Abita (INSERM, Paris) [22]. Treatment of the human myelomonocytic leukemic cell line HL-60 with all-*trans*-retinoic acid, or with specific retinoic acid analogues, induced the appearance of terminally differentiated myeloid cells. Early events in this process were observed to be an elevation of intracellular Na^+, which stimulates the sodium pump, and a rise in intracellular pH, mediated by Na^+/H^+ exchange. The long-term alkalinization of the cell from pH 7 to 7.37, as measured by intracellular pH-sensitive dyes, was blocked by ethylamiloride. Retinoic acid changed the maximal velocity of the Na^+/H^+ antiporter by a mechanism which remains unclear; structure–activity studies amongst retinoic acid analogues suggest that there are discrete receptors for the retinoids at the level of the membrane [23], although whether these are related to the recently discovered nuclear retinoid receptor [24] is unclear as attempts to isolate the membrane receptor have failed. Furthermore, there was no evidence that the Na^+/H^+ antiporter was activated by protein kinase C activation via the release of diacylglycerol; on the contrary, a decrease in diacylglycerol production was reported. Although a clearer understanding of the intricate mechanisms involved in the promotion of differentiation by retinoids, and other membrane-active agents such as the polar solvents, may lead to better drugs, Dr Abita closed by describing the developing success of the strategy of differentiation therapy in the treatment of acute human promyelocytic leukaemia with retinoic acid.

The achievement of selectivity of drug action is a central concept of cancer chemotherapy. Dr Roger Camble (ICI, Macclesfield) reviewed the strategies open to the biotechnologist, capitalizing on the specificity of biological proteins provided by millions of years of evolution. He concentrated on the growth factors and their receptors, and outlined progress in the discovery of small molecular weight antagonists and of antibodies. The 'small' compounds arose from the synthesis of modified peptides, such as those based on bombesin in which methylene groups may be introduced, and higher molecular weight compounds which have arisen from the synthesis of genes. Three-dimensional models of growth factors, such as the high resolution NMR prediction of the stereochemistry of EGF [25] facilitate the engineering of such antagonists. Antibody diversity means that, potentially, 10^9 agents may be isolated, some of which would have high specificity of binding for growth factor receptors: antibodies to the EGF receptor have an

affinity of 10^{-10} M. Their drawbacks are immunogenicity and the problems of delivery. The immunogenicity may be engineered out by generating minimal fragments, or the replacement of the constant region by a human derived isotype. Minimalization of the V_H and V_L regions and their joining by synthetic peptides is an alternative strategy. The other advantage to the minimalized fragments which recognize growth factor receptors is that they may be used in computer graphics modelling in the search for small, organic chemicals which may, in the long-term, be pharmacokinetically preferable to the products of biotechnology.

The ether lipids, 1-*O*-alkyl-lysophospholipids, the activity of which Dr Wolfgang Berdel (Munich) reviewed, undoubtedly were molecules with a chemical affinity with membranes. The prototype, Et-*O*-18Me (1-*O*-octadecyl-2-*O*-methyl racemic glycero-3-phosphocholine) and a host of analogues have *in vivo* activity against a variety of murine tumour models, and against the metastatic Lewis lung carcinoma [26]. The mode of action of this class of compound is unclear: currently it is proposed that tumour cell kill is mediated indirectly via the activation of macrophage and/or elements of direct cytotoxicity. Dr Berdel showed evidence that treatment of human brain tumour cells with Et-*O*-18Me brought about cell surface blebbing, and that after 48 h *in vitro*, electron micrographs showed membrane rupture and discrete pitting of the cell surface. What is not yet defined is how this damage arises: whether from physical disruption of the membrane bilayer and cytoskeleton, or whether potentially more selective biochemical mechanisms are involved. Clearly, from the screening data, elements of selectivity reside in the ether lipids and Dr Berdel was optimistic with regard to the generation of even more selective molecules and to the use of extended structure–activity studies for the determination of minimal structural requirements for activity [27]. Clinically, certain compounds are already showing promise in the purging of leukaemic bone marrows [28], although the preliminary results suggested a surprising degree of donor variation, with regard to sensitivity.

Finally, Dr Bang Luu (Strasbourg), introduced another class of membrane active agents which are in their development stage: the 7-β-hydroxy steroids [29]. Their rather selective cytotoxic activity (toxic to HTC cells but not to 3T3 cells) is antagonized *in vitro* by cholesterol and lipoproteins and, similarly to the ether lipids, physical membrane damage, including cell surface blebbing and changes in membrane fluidity, were reported. Dr Luu also outlined some functional changes in lymphocytes after administration of 25-hydroxycholesterol: it blocked Con-A activation of lymphocytes, an activity prevented by incubation with high potassium concentrations. Additionally, the production of IL-2 was inhibited. The evidence suggested that these chemically modified steroids interact with the plasma membrane to disrupt cellular signalling mechanisms.

In a recent review of the activity of present-day antitumour drugs on membrane activity, most drugs were reported to modify the structure and/or function of the plasma membrane [30]. The emergence of new chemical entities, such as the ether lipids, and an increasing understanding of the physiology and biochemistry of the control of cell differentiation and proliferation suggest that the understandably limited perception of potential targets for antitumour drugs has diminished. Many of the problems of current chemotherapy—tumour heterogeneity, poor vascularization, the development of resistance and low growth fraction tumours—will remain irrespective of the medicinal chemists' ingenuity. It is nevertheless important that the armamentarium of drugs increase, and that investigations of the biochemistry and pharmacology of cell death and the promotion of cellular differentiation encompass the whole of cell physiology. It was agreed by the participants of this membrane targets meeting that this process had begun.

Acknowledgements—The EORTC-PAM group wish especially to thank Dr Gerard Milano in Nice for excellent local organization, and Glaxo (U.K.), ICI (U.K.), Roche (Switzerland), Farmitalia (Italy), Boehringer-Mannheim GmbH (F.R.G.) and Smith Klyne and French (U.K. and U.S.A.) for generous financial support.

REFERENCES

1. Cohen P, Houslay M (eds). *Molecular Mechanisms of Transmembrane Signalling*, Vol. 4. In: *Molecular Aspects of Cellular Regulation*, Elsevier, New York, 1985.
2. Rozengurt E. Early signals in the mitogenic response. *Science* 1986, **234**, 161–166.
3. Bishop M. The molecular genetics of cancer. *Science* 1986, **235**, 305–311.
4. Moolenaar WH. Effects of growth factors on intracellular pH regulation. *Ann Rev Physiol* 1986, **48**, 363–376.
5. Moolenaar WH, Kruijer W, Tilly BC, Verlaan I, Bierman AJ, de Laat SW. Growth factor-like action of phosphatidic acid. *Nature* 1986, **232**, 171–173.
6. L'Allemain G, Paris S, Pouyssegur J. Role of a Na^+-dependent Cl^-/HCO_3 exchange in regulation of intracellular pH in fibroblasts. *J Biol Chem* 1984, **259**, 5809–5815.
7. Chambard JC, Paris S, L'Allemain G, Pouyssegur J. Two growth factor signalling pathways in fibroblasts distinguished by pertussis toxin. *Nature* 1987, **326**, 800–803.
8. Paris S, Pouyssegur J. Growth factors activate the bumetanide-sensitive $Na^+/K^+/Cl^-$ cotransport in hamster fibroblasts. *J Biol Chem* 1986, **261**, 6177–6183.

9. Parker PJ, Ullrich A. Protein kinase C. *J Cell Physiol* 1987, Suppl. 5, 53–56.
10. Akiyama T, Ishida J, Nakagawa S *et al.* Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J Biol Chem* 1987, **262**, 5592–5595.
11. Varshavsky A. Phorbol ester dramatically increases incidence of methotrexate-resistant mouse cells: possible mechanisms and relevance to tumour promotion. *Cell* 1981, **25**, 561–572.
12. Bojan F, Kinsella AR, Fox M. Effect of tumor promotor 12-*O*-tetradecanoylphorbol-13-acetate on recovery of methotrexate-, *N*-(phosphonacetyl)-L-aspartate- and cadmium-resistant colony-forming mouse and hamster cells. *Cancer Res* 1983, **43**, 5217–5221.
13. Szallasi A, Fox M, Kinsella AR. The enhancement of the recovery of methotrexate and *N*-phosphonacetyl L-aspartate resistant mouse 3T6 cell clones is associated with transient alterations of cell cycle progression. *Int J Cancer* (in press).
14. Grace T, Kimberly P, Hacker M, Tritton TR. Stimulation of growth of Adriamycin. *Proc Am Assoc Cancer Res* 1987, **28**, 266.
15. Zuckier G, Tritton TR. Adriamycin causes up-regulation of epidermal growth factor receptors in actively growing cells. *Exp Cell Res* 1983, **148**, 155–161.
16. Thompson MG, Chahwala SB, Hickman JA. Inhibition of human erythrocyte inositol lipid metabolism by Adriamycin. *Cancer Res* 1987, **47**, 2799–2803.
17. Ross WE. DNA topoisomerases as targets for cancer therapy. *Biochem Pharmacol* 1985, **34**, 4191–4195.
18. Grunicke H, Gantner G, Holzweber F, Ihlanfeldt M, Puschendorf B. New concepts on the interference of alkylating antitumour agents with the regulation of cell division. *Adv Enzyme Reg* 1979, **17**, 291–305.
19. Doppler W, Hofmann J, Oberhuber H, Maly K, Grunicke H. Nitrogen mustard interference with potassium transport systems in Ehrlich ascites tumor cells. *J Cancer Res Clin Oncol* 1985, **110**, 35–41.
20. Grunicke H, Doppler W, Hoffman J *et al.* New strategies for the improvement of alkylating antitumor agents. In: Cory JG, Szentivanyi A, eds. *Cancer Biology and Therapeutics*. New York, Plenum Press, 1987, 127–139.
21. Waxman S, Rossi G, Takaku F (eds). *The Status of Differentiation Therapy of Cancer*. Sereno Symposium Publication, Raven Press, New York, 1988.
22. Ladoux A, Cragoe EJ, Geny B, Abita J-P, Frelin C. Differentiation of human promyelocytic HL60 cells by retinoic acid is accompanied by an increase in the intracellular pH. *J Biol Chem* 1987, **262**, 811–816.
23. Chomienne C, Balitrand N, Cost H, Degos L, Abita J-P. Structure–activity relationships of aromatic retinoids on the differentiation of the human histiocytic lymphoma cell line U-937. *Leuk Res* 1986, **10**, 1301–1305.
24. Petkovich M, Brand NJ, Krust A, Chambon P. A human retinoic acid receptor which belongs to the family of nuclear receptors. *Nature* 1987, **330**, 444–450.
25. Cooke RM, Wilkinson AJ, Baron M *et al.* The solution structure of human epidermal growth factor. *Nature* 1987, **327**, 339–341.
26. Berdel WE, Munder PG. Antineoplastic actions of ether lipids related to platelet-activating factor. In: Snyder F, ed. *Platelet-Activating Factor and Related Lipid Mediators*. New York, Plenum Press, 1987, 449–467.
27. Danhauser S, Berdel WE, Schick HD *et al.* Structure–cytotoxicity studies on alkyl lysophospholipids and some derivatives in leukemic blasts of human origin. *Lipids* 1987, **22**, 911–915.
28. Okamoto S, Olsen AC, Vogler WR. Elimination of leukemic cells by the combined use of ether lipids *in vitro*. *Cancer Res* 1987, **47**, 2599–2603.
29. Hietter H, Bischoff P, Beck JP, Ourisson G, Luu B. Comparative effects of 7-β-hydroxycholesterol towards murine lymphomas, lymphoblasts and lymphocytes: selective cytotoxicity and blastogenesis inhibition. *Cancer Biochem Biophys* 1986, **9**, 75–83.
30. Tritton TR, Hickman JA. Cell surface membranes as a chemotherapeutic target. In: Muggia FM, ed. *Chemotherapy*, Vol. 2, Martinus Nijhoff, The Hague, 1985, 81–131.