NUEVAS DIANAS MOLECULARES TERAPÉUTICAS EN LAS NEOPLASIAS MALIGNAS
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Resumen del simposio

En los últimos años hemos asistido al desarrollo de nuevas moléculas que actúan frente a dianas terapéuticas específicas. Este campo de la investigación ha aportado soluciones a los enfermos diagnosticados de hemopatías malignas y es evidente que continuará progresando. Al tratamiento con ATRA de la leucemia aguda promielocítica se ha añadido el uso del mesilato de imatinib en la terapia de la leucemia mieloide crónica y de los síndromes mieloproliferativos con afectación de genes con actividad tirosinquimasa (PDGFR alfa y beta, entre ellos) y un cada vez mayor número de anticuerpos monoclonales, que han demostrado su utilidad en el tratamiento de los linfomas. Por ello, hemos organizado este simposio en el que hemos pretendido aunar la actividad de la investigación básica y de la aplicada y contemplar el uso de estos fármacos frente a síndromes mio y linfoproliferativos. El Dr. Faustino Mollinedo es investigador del Centro de Investigación del Cáncer de la Universidad de Salamanca-CSIC y ha desarrollado una importante labor en el estudio del mecanismo de acción de nuevos fármacos antitumorales, con especial énfasis en su actividad reguladora de la apoptosis. Su ponencia se centrará en el análisis de las cualidades que debe tener un fármaco para poder ser usado en la práctica clínica como antineoplásico a la vez que analiza con una visión integradora los avances y la complementariedad de las nuevas técnicas genómicas, bioinformáticas y de análisis funcional. La Prof. Mele, trabaja en el Hospital Hammersmith de Londres y ha realizado importantes avances en la investigación del uso del mesilato de imatinib en la leucemia mieloide crónica. Su trabajo versará sobre la definición de nuevas dianas terapéuticas en el tratamiento de las hemopatías mieloideas. El Dr. Caligiuri pertenece a la División de Hematología del Hospital de Ohio y presentará los datos relativos uso de nuevas moléculas para el tratamiento de los síndromes linfoproliferativos asociados a infección por virus de Epstein-Barr, cada vez más frecuentes en enfermos inmunodeprimidos. En su trabajo el uso de la inmunoterapia como complemento de la retirada de la inmunodepresión ofrece posibilidades esperanzadoras en el tratamiento de los linfomas postrasplante. Finalmente el Dr. Cortés, del MD Anderson de Houston explicará cuáles pueden ser las soluciones en los casos de resistencia al mesilato de imatinib. Con estas cuatro aportaciones de especialistas de primer nivel en el campo de los tratamientos diseñados frente a dianas específicas se afianzará nuestro conocimiento de esta auténtica revolución en la terapia neoplásica.
INTEGRATION OF BIOLOGICAL CONCEPTS, GENOMICS AND HIGH-THROUGHPUT TECHNOLOGIES IN CANCER DRUG DISCOVERY

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Abstract
Advances in high-throughput molecular technologies, including genomics, proteomics and microarrayed compound screenings, have led to a dramatic change in the way of how drugs are discovered. These technology breakthroughs together with our growing understanding of the molecular aspects of cancer development and treatment are leading to the advent of novel therapeutic strategies and to the generation of new drugs to fight against cancer. In addition new frameworks are being set up in the pursuit of more specific and selective anticancer drugs. Likewise, the use of genomic-scale approaches is leading to a more complete knowledge and definition of cancer at the molecular level, and this will entail to classify and characterize new distinct cancer subtypes as well as to target them more specifically. Furthermore, our current understanding of apoptosis as well as its implication in cancer development and treatment should facilitate the design of new therapies. These apoptotic-targeted approaches, hopefully, may improve the clinical outcome of cancer therapy, especially for those tumours with a low growth capacity.

Conceptual basis and molecular targets for cancer therapies
There are several strategies for identifying new chemical entities, either synthesized chemicals or natural compounds extracted or derived from plant, microbial or marine sources. The history of cancer drug discovery reflects an evolution from highly empiric approaches, based on testing of randomly selected compounds against rapidly proliferating murine leukaemia, to the current, more focused testing of natural products, rationally synthesized agents, and biologic products against well-characterized tumour cell lines or molecular targets. In addition, the advent of novel high-throughput molecular and biologic technologies, including genomics and proteomics, together with microarrayed compound screening (μARCS™) technologies, have led to a dramatic change in the way of how drugs are discovered and to speed up the pace in finding a particular drug for a specific target.

Throughout history drug discovery has been largely influenced by the prevailing ideas and paradigms of each period of time (table 1). Thus the predominant paradigms of the 1950s to 1970s considered that: a) tumours were composed of rapidly dividing cells; b) tumour cells lost the normal growth control that regulate progression through cell cycle, leading to the notion that cancer cells are continuously cycling and dividing, and c) cancer cells became somehow immunologically “foreign” or dedifferentiated so as to present cancer-specific antigens that could be exploitable via immunological mechanisms. Later, in the 1980s to 1990s the knowledge of the molecular changes in oncogenes, tumour suppressor genes, and the diverse set of genes that control senescence, anogenessis, and apoptosis during tumour formation led to define new targets for cancer drug discovery. In this regard, gain-of-function mutations in oncogenes (Ras, Abl, Myc, epidermal growth factor receptor -EGFR-) led to the development of small-molecule inhibitors of the corresponding signalling events (e.g., farnesyltransferase inhibitors to block Ras activation). On the other hand, loss-of-function mutations or deletions in tumour suppressor genes (Rb, p53) led to gene therapy approaches trying to restore the mutated key cellular sensor. In addition, a number of evidences showed that tumour cells exhibit telomerase activity, adding new telomeric DNA to the ends of chromosomes replacing the sequences lost during repeated rounds of cell division, and thereby the development of telomerase inhibitors could eventually block tumour proliferation. In the late 1990s an indirect and ingenious way to target cancer relied on the fact that tumour cells induce neovascularization of tumours (anogenessis), as they require new blood supplies to maintain viability. The synthesis of a number of key molecules, including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and integrins, resulted essential for this process, and therefore a series of anogenessis inhibitors (TNP-470, suramin) started to be developed. Furthermore, it also became clear that tumour cells possess defects in their DNA repair process, resulting in increased frequency of random mutations. This leads to the hypermutability or plasticity of some cancer phenotypes that ultimately contributes to the acquired drug resistance phenotype. This genetic instability is a major problem in the successful treatment of cancer.

Genomic-scale approaches and pharmacogenomics
With the beginning of the twenty first century a burst in high-throughput technologies have allowed us to improve and speed up our way to identify novel targets and to generate new target-specific drugs. The advent of DNA microarray technology as well as other high-throughput techniques have revolutionized both the way to face drug discovery as well...
as to classify disease, which will translate, very likely, into a new way to treat patients. All marketed drugs so far target about 500 gene products\(^1\,^2\). Thus, it is expected that a great number of the 30,000-40,000 protein-coding genes in the human genome, will offer immense new opportunities for the identification of new potential targets.

The development of cancer is accompanied by genomic alterations of multiple genes, especially oncogenes and tumour suppressor genes. Before the genomic onset, the standard techniques of molecular biology have been used to identify a limited number of genes in the cancer development process. However, these methods are highly focused, targeting one or few genes at a time, and do not provide broad insight into global changes in gene expression patterns. With the advent of DNA microarrays, it is possible to survey the level of expression of thousands of genes at a time, leading to unforeseen data involving unexpected genes in the drug discovery process. Thus, DNA microarray is a promising tool for monitoring complex gene expression patterns required to be analysed in complex diseases, like cancer, that involve the participation of a rather wide number of genes.

### Table 1. Conceptual Basis and Molecular Targets for Cancer Therapeutics

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<th>Biologic concept</th>
<th>Molecular targets</th>
<th>Therapeutics</th>
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<td>Rapidly dividing cells, loss of cell growth control</td>
<td>Nucleic acids, proliferative machinery, DNA synthesis, microtubules, mitotic spindle apparatus</td>
<td>Alkylation agents (cyclophosphamide, busulfan), Topoisomerase inhibitors (etoposide, doxorubicin), Antibiotics (mitomycin C, actinomycin D), Antimetabolites (cytosine arabinoside, mercaptopurine, methotrexate, fludarabine, fluorouracil), Microtubule-interfering agents (vincristine, vinblastine, vinorelbine, paclitaxel, docetaxel)</td>
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<td>Immunologically “foreign” or dedifferentiated cells</td>
<td>Tumor selective antigens, Nuclear receptors</td>
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<td>Activated oncogenes and critical protein kinases</td>
<td>Ras GTP binding proteins, BCR-ABL kinase, Src kinase, EGFR, PDGFR, c-KIT, VEGF, Raf kinase, Cyclin-dependent kinases</td>
<td>Farnesyl transferase inhibitors, Tyrosine kinase inhibitors (Gleevec(^\text{TM})–STI571–, Iressa(^\text{TM})–ZD1839–, Imatinib–SU11274–, Sorafenib)</td>
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<td>Loss of tumor suppressor genes</td>
<td>Retinoblastoma protein, p53, AT, MCC, APC, von Hippel-Lindau protein</td>
<td>Gene therapy to restore normal suppressor gene function</td>
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<td>Abnormal DNA repair mechanisms</td>
<td>DNA mismatch repair enzymes–MSH2, MLH1, PMS1, PMS2</td>
<td>Gene therapy to restore normal enzyme activity, Checkpoint inhibitors to promote susceptibility to DNA damaging agents</td>
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<td>Epigenetic inactivation of genes</td>
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<td>DNA methyltransferase (DNMT) inhibitors (5-azacytidine and 2′-deoxy-5-azacytidine –Decitabine–, Histone deacetylase (HDAC) inhibitors (suberoylanilide hydroxamic acid –SAHA–)</td>
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\(^1\)\(^2\) XLVI Reunión Nacional de la AEHH y XX Congreso Nacional de la SETH. Simposios
cogenomics, that holds the promise of improving dramatically the efficacy and reducing the risk of drug treatment by identifying markers that predict an individual’s response to a drug. A major goal of pharmacogenomics is to elucidate the association between genetic variation of an individual and drug response, thereby identifying genetic markers that predict response to drugs in the clinic. This involves the identification of the level of expression of genes responsible for drug absorption and metabolism as well as target genes for a particular drug in each individual together with the expression level of those genes involved in the signalling pathways mediating the drug effect. Pharmacogenomic markers also include both indicators of DNA differences between individuals (e.g., SNPs) as well as quantitative differences between gene expression levels of normal and tumour tissues. Altogether, implementation of these techniques will lead to what is called "personalized medicine", in which the association of drug response with molecular markers will open new opportunities for drug discovery in non-responding patients and, more important, distinguish the sort of most convenient drug for each individual because of a higher confidence in expected efficacy and safety (fig. 1).

In addition, the use of genomics in pharmaceutical research will help to elucidate the mechanism of action of anticancer drugs, to provide novel high-quality targets as well as to reduce the percentages of preclinical and clinical failures. Nevertheless, most of these failures are due to the fact that the current in vitro and in vivo models used to evaluate antitumor activity are faulty and lack predictability, and hence the generation of novel more reliable models for antitumor activity is much needed.

Our increased understanding of the genomics and molecular pathology of cancer provides us with an intellectual framework for devising innovative and ingenious strategies to strive it (fig. 1). Thus, identification of new targets driving the molecular pathology and progression of human cancer will provide a framework for discovering new target-directed drugs with a putative improved efficacy and selectivity. This is much needed due to the lack of selectivity of the current antitumor agents, which is another major problem in cancer chemotherapy, and leads to compounds with a rather poor therapeutic index, defined as the ratio of maximum tolerated dose/minimum effective dose. Medically useful drugs should ideally have a high therapeutic index. The combination and implementation of new technologies such as genomics, proteomics, high-throughput screening (HTS), combinatorial chemistry, structural biology and computational modelling, are largely contributing to accelerate the pace and efficiency of drug discovery (fig. 1).

New targets must be validated in order to assure its importance in cancer development and/or treatment. Target validation includes several factors, although not all of them must be met to get on a drug discovery program: a) assessment of the frequency of genetic or epigenetic deregulation of the target or signalling pathway in human cancer; b) demonstration in model systems, both in vitro and in vivo, that the target contributes to the malignant phenotype; c) evidence for the reversal of the malignant pheno-
type blocking or silencing the target gene; and d) feasibility of designing a drug for the specific target. Both over-expression and gene-deletion technologies are useful in assessing target validation. However, because most small-molecule drugs achieve antagonism rather than agonism of an effector, gene deletion technology is favoured. Thus, mammalian gene-silencing and knockout technologies including antisense oligonucleotides, small interference RNAs (siRNA) and knock-out in mice are widely used. Finally, as mentioned above, the target must be feasible for drug development. In this regard enzymes are generally more tractable than are most protein-protein interactions.

In addition, the analysis of thousands of genes at a time during oncogenesis, drug resistance and drug treatment is leading to the development of diagnostic, prognostic and pharmacogenomic biomarkers to enable the targeting of individualized treatments to those patients most likely to benefit. This is leading to a new concept in cancer therapy, redefining and reclassifying cancer in a number of increasing molecular mechanisms in particular malady subtypes, leading to redefine diseases with greater accuracy at the molecular level and classify diseases, select patients for specific treatments, and develop tests for diagnosis and prognosis. In fact, the term cancer includes more than 200 distinct entities.

Targeted treatment in specific disease subtypes

Most drugs work, at best, for 60% of the patients for whom they are prescribed, and drugs that work well for some patients make others ill. This is largely due to individual variations in metabolism and/or to a deficient definition of different disease subtypes. Use of genomic and proteomic techniques will allow to characterize different disease pathologies and molecular mechanisms in particular malady subtypes, leading to redefine diseases with greater accuracy at the molecular level and classify diseases, select patients for specific treatments, and develop tests for diagnosis and prognosis. In fact, the term cancer includes more than 200 distinct entities.

Molecular approaches (DNA microarrays and proteomics) are being widely used to define and classify disease subtypes more accurately, such as cancer8,9, separating in this way diseases that are currently clustered together as if they were the same disease, and treat them as clinically defined subtypes within a disease family. Thus, genomics and proteomics are expected to lead to a) development of tests to identify people with specific metabolic variations and disease subtypes; b) identification of suitable targets, and subsequent validation; and c) development of diagnostics and monitoring tests based on the molecular markers of the disease subtypes to evaluate progression, severity and efficacy outcome. In addition, redefining cancer subtypes with great accuracy at the molecular level will make it considerably easier to validate a target. For instance, overexpression of the HER2/neu (c-erbB2) gene occurs in various cancers, including 20% of breast cancers, has led to define a cancer subtype and to develop a drug specifically designed for these patients, Herceptin™ (Genentech). A number of spin-offs have subsequently come out, such as a test to identify patients who overexpress the HER2/neu protein.

It is now expected that more than three decades of research on molecular aspects of cancer development and treatment may finally pay off in a new generation of more specific, less toxic, cancer drugs.

Gleevec as a drug discovery paradigm for a specific type of cancer

Gleevec™ (also named Clivex, imatinib mesylate, and STI571, formerly CGP57148), manufactured by Novartis (Basel, Switzerland), exemplifies the successful development of a rationally designed, molecularly targeted therapy for the treatment of a specific cancer, the chronic myelogenous leukaemia (CML). This disease is a clonal hematopoietic stem cell disorder associated, in more than 95% of cases, with the (9;22) chromosomal translocation that involves the long arms of both chromosomes, thereby generating the known Philadelphia (Ph) chromosome, a hallmark of this disease, joining the bcr gene in chromosome 22 to the abl gene from chromosome 9. The molecular consequence of this event is the generation of a chimeric bcr-abl gene that codes for a 210-kDa protein, which is seen in 95% of patients with CML. Abl is a tyrosine kinase involved in cell signalling and once the chimeric protein, consisting of the N-terminal fragment of Bcr fused to the C-terminal of Abl, is formed the tyrosine kinase activity is constitutively hyperactive, so that it stimulates proliferation of the Ph leukaemia cells and inhibits these cells from dying by apoptosis, leading to excessive numbers of white cells that are released into the bloodstream, producing leukaemia. Transduction of p210 Bcr-Abl into murine hematopoietic stem cells, causes a CML-like syndrome8,9. Therefore, the chimeric Bcr-Abl protein became an obvious target for therapeutic attack. Thus, after being well known that the tyrosine kinase activity of the Bcr-Abl quimeric protein was the cause of CML, Brian J. Druker and Ciba Geigy (now Novartis) in 1990 conducted a series of assays searching for a compound able to inhibit specifically this activity. Of the several compounds generated Gleevec emerged as the lead compound for clinical development based on its high in vitro selectivity against CML cells. In the summer of 1998 a phase I study was initiate and 54 patients that had failed therapy with interferon α were treated with Gleevec. The results were surprising, 53 out of 54 patients achieved a complete haematological response10,11. The excellent results obtained with this drug in CML treatment has turned this drug as a new paradigm in drug discovery.

On the other hand, Gleevec is also known to inhibit two additional tyrosine kinases, namely the platelet-derived growth factor receptor (PDGFR) and stem-cell factor receptor (c-Kit), which are be...
Hsp90 client proteins include prepared in a wide variety of cancer cells and in virally transformed ones (Heat shock proteins, Hsps), required for the stability and function of multiple signalling molecules that promote growth and survival of cancer cells. One of the most abundant Hsps is Hsp90. By the early 1990s, several observations showed that Hsps in general, and Hsp90 in particular, were over-expressed in a wide variety of cancer cells and in virally transformed cells. Hsp90 client proteins include mutated p53, Bcr-Abl, Akt, ErbB2, and is constitutively expressed at 2-10-fold higher levels in tumour cells compared with their normal counterparts. Thus, targeting Hsp90 is expected to affect a number of gene products important for cancer cell survival. Geldanamycin and the benzoquinone ansamycin 17-allylamino geldanamycin (17AAG) are potent Hsp90 inhibitors with potential antitumor activity. A hallmark of cancer cells is the epigenetic inactivation of genes that are crucial for the control of normal cell growth (Rb, p16, APC, caspase-8, DNA repair genes, etc). Epigenetic transcriptional repression of genes include DNA methylation and histone deacetylation and methylation. In unmethylated nucleosomal DNA, histone acetylation leads to active transcription of genes by RNA polymerase II, however methylation of CpG islands in DNA near to gene promoters is associated with chromatin changes such as deacetylation and methylation of core histones, leading to exclusion of transcription factors and subsequently to gene silencing. Histone deacetylases (HDAC) inhibitors (butyrates, trnopaxin A, apicidin, benzamides, and hydroxamic acids, such as trichostatin, oxamflatin and suberoylanilide hydroxamic acid -SAHA-) and DNA methyltransferase (DNMT) inhibitors (5-azacytidine, 2′-deoxy-5-azacytidine, also known as Decitabine) restore expression of genes silenced by methylation in cancer cells, promoting growth arrest, cell differentiation and apoptosis. Another target that controls ultimately the expression of plenty of survival regulators is the proteasome. The 26S proteasome degrades proteins that have been marked by the addition of multiple ubiquitin molecules, a process called ubiquitination, to short (3-22 residue) polyptides. Thus, targeting proteasome could have a potential for the treatment of cancer due to the decrease in the level of survival gene products. The first compounds identified as proteasome inhibitors included the natural inhibitor lactacystin and synthetic peptide aldehydes related to calpain inhibitor I. Further modifications in the structure of these latter compounds, and the differential effect that proteasome inhibition exerts on malignant cells, have led to the development of the dipetidyl boronic proteasome inhibitors, including PS-341. PS-341 (borzomb, Velcade) proteasome inhibitor shows promising anticancer activity in both haematological, especially multiple myeloma, and solid tumour malignancies. Apoptosis as a major cancer therapeutic target

The only modest progress in cancer chemotherapy over the past sixty years suggests that some of the frameworks used for targeting the cancer cell are wrong. Perception of the malignant cell as having only an uncontrolled proliferation is one of such frameworks. The rather modest impact of antiproliferative drugs in clinic is not surprising since many tumours have a low growth capacity. In contrast, increasing evidence is leading to the conception of a tumour cell as a cell mainly defective in triggering its own death by apoptosis. In addition, it is becoming clear that malignant tumours are comprised of both cancer stem cell, with a high proliferative potential, and more differentiated cancer cells, with limited proliferative potential. A number of cancer stem cells have been identified that have the potential to self-renew, to transfer disease and to form tumours following transplantation. Stem cancer cells can be envisioned to be rather resistant to apoptosis. Thus, this could explain the frequent failure of chemotherapy in curing cancers. More differentiated cancer cells could be killed by chemotherapy leading to tumour shrinkage, but if cancer stem cells survive, these latter will continue the process of tumour growth and progression. Thus, new approaches should be developed in order
to efficiently promote killing of stem cancer cells or differentiation into a more drug-sensitive cell or a be-nign tumour, exhausting the pool of cancer stem cells.

Anticancer agents with diverse primary targets, in-cluding microtubule-active agents, topoisomerase II inhibitors, DNA-alkylating agents, antibiotics and fo-late antagonists, trigger signalling pathways leading ultimately to apoptosis in a rather indirect way (fig. 2). Conversely, because drugs with different targets induce apoptosis through similar ultimate apoptotic mechanisms, mutations in apoptotic programs lead to multidrug resistance and treatment failure. Thus, the effectiveness of anticancer drugs reflects the cell’s ability to detect and respond to the perturbation induced by the drug. The majority of anticancer drugs target DNA synthesis, whereas others affect cellular metabolism and cell division. In this re-gard, induction of apoptosis by these drugs is rather indirect because effects on cell cycle or cell metabol-ism must be sensed by the cells as signals that, after reaching a certain threshold, trigger an apoptotic re-sponse. Thus, cells sense this drug-induced damage or cellular perturbation through the presence of “sens-ors”, calibrate these lesions and mount a response to these aggressions, reacting according to their pheno-type. When this drug-induced metabolic, bio-chemical or DNA damage is excessive or difficult to be repaired, the cell decides to self-destruct by apop-tosis avoiding in this way to threaten adjoining cells and tissues. The failure of some tumour cells to die following a chemotherapeutic treatment may be due to either their inability to sense the drug-promoted harm or their resistance to engage apoptosis. This implies the existence of an “apoptosis threshold”, in response to damage that is set differently in distinct cell types. Mutations in sensors involved in trigger-ing an apoptotic response hamper the onset of apop-tosis, and it is well-known that cancer cells try to get rid of sensors that can lead to cell death enhancing their “apoptosis threshold”. In this regard, the tu-mour suppressor gene p53, involved in cell-cycle con-trol, apoptosis, and in the maintenance of genetic stability, is mutated in about half of all human can-cers. In order to avoid putative mutations in the sen-sors linking cell damage to a cell death response that could hinder or frustrate the use of anti-cancer thera-pies, a direct activation of the apoptotic machinery should be more appropriate to kill rapidly and effi-ciently the tumour cells. The idea of developing drugs able to target and induce apoptosis in tumour cells constitutes an attractive and promising ap-proach in cancer treatment. Direct activation of the cell death machinery could circumvent the action of cellular sensors and checkpoints, which are frequent-ly mutated or altered in cancer, and therefore their mutant state should be largely irrelevant to this ther-apecutic approach (fig. 2).

Three major components of the cell death machin-ery include: death receptors, regulators (Bcl-2 family members), and executors (caspases). Likewise two major signalling pathways play critical roles in the trig-gering of apoptosis, namely the extrinsic pathway (death receptor-mediated route) and the intrinsic pathway (mitochondria-mediated route), leading to the formation of two major apoptotic complexes: DISC (death-inducing signalling complex), made up of Fas, Fas-associated death domain protein (FADD) and procaspase-8, and apoptosome, made up of cytochrome c, Apaf-1 and procaspase-9. A number of new drugs and biological substances are being de-veloped to promote killing of cancer cells in a rather direct way, namely by promoting oligomerization of Apaf-1 into the apoptosome through indolone com-pounds or by antagonizing the ant apoptotic func-tion of Bcl-2 or Bcl-XL with the low molecular weight organic compound HA14-1, discovered using a com-puter screening strategy, or the natural products Tetrocarcin A and Antimicyn A. In addition, our recent demonstration of the translocation and cap-ping of the death receptor Fas (also known as APO-1 or CD95) into membrane rafts in Fas-mediated apop-tosis, subsequently confirmed by other investi-gators, has led to another new efficient way to trigger apoptosis in tumour cells. Thus, cyto-toxic drugs can be used in chemotherapy via enhance-ment of raft dependent killing of tumour cells. Such as a notion has found experimental support in our recent studies in which the antitumour ether lipid ET-18-OCH3 induces apoptosis in tumour cells via

Figure 2. Induction of apoptosis in current cancer chemotherapy involves a multi-step pathway. Antiproliferative drugs induce a cell damage into the cancer cell that is sensed through damage sensors. These latter activate a number of signalling routes, some of those eventually trigger apoptosis. Mutation or deletion of damage sensors in tumours precludes or hampers the triggering of downstream signalling events. A direct activation of the apoptotic signalling could hypothetically circumvent these obstacles and lead to rapid demise of cancer cells.
capping of Fas-containing raft membrane microdomains. This capping presumably amplifies raft-dependent Fas signalling, likely through structural reorganization of raft membrane microdomains. Our data also indicate that rafts represent a potential target for therapeutic intervention in cancer.

Outlook

Advances in elucidating the molecular mechanisms involved in cancer development and treatment, greatly facilitated by the outbreak of novel high-throughput technologies, will lead to an outburst in the choices of drug discovery targets in the future. With the aid of genomics and proteomics, it is expected that cancer will be more precisely subdivided into different disease subtypes with remarkable and specific molecular features. Some of these molecular peculiarities or hallmarks might become major therapeutic targets that, in turn, could result in the development of new more specific and selective drugs for each cancer subtype. Redefining cancer subtypes with greater accuracy as the molecular level makes it considerably easier to validate a target, and putatively increases the efficacy outcome of a target-directed therapy. In addition, direct activation of the apoptotic machinery in cancer cells opens a new framework in drug discovery, averting the deleterious effect of a great number of mutated sensors, inherent to the genetic instability of cancer cells, that frequently hampers the use of DNA-targeted and antiproliferative drugs.

References

access to the human host via primary infection of the epithelial cells of the nasopharynx. Here, activation of an EBV lytic gene program results in local virus production and infiltration of submucosal lymphoid tissue where naïve/resting B lymphocytes are infected. It is primarily within this resting B-cell compartment that EBV establishes a persistent, subclinical infection. This is achieved via a highly coordinated EBV latent gene expression program which downregulates the expression of immunogenic EBV peptides in response to an efficient T-cell mediated immune response to the virus. EBV is associated with a variety of neoplastic diseases including lymphoproliferative disorders (LPD) that arise in patients with congenital (X-linked-LPD), acquired (AIDS-lymphoma), or iatrogenic (post transplant lymphoproliferative disorder, PTLD) immune deficiency, Burkitt’s lymphoma (BL), Hodgkin’s disease (HD), a subset of T/NK-cell lymphomas (T/NK-cell lymphoma nasal type), and lymphoepithelioid carcinomas of the gastric and nasopharyngeal mucosa.

Each EBV-associated malignancy is characterized by a specific pattern of EBV gene expression. Three major latent gene programs have been described (fig. 1). EBV+ resting B cells and Burkitt Lymphoma (BL) cells display a Latency I gene expression profile restricted to the expression of the EBV nuclear antigen 1 (EBNA1) and Latent Membrane Protein-2 (LMP-2). Hodgkin’s disease (HD), T/NK-cell lymphomas and Nasopharyngeal Carcinomas (NPC) express a Latency II gene expression profile restricted to expression of EBNA1, LMP-2 and LMP-1. Finally, LPD associated with immune deficiency and EBV-transformed human B-cells capable of proliferating indefinitely in-vitro (Lymphoblastoid Cell Lines, LCL), display a less restricted Latency III profile and express EBNA1, EBNA-2, EBNA-3a, 3b, 3c, EBNA-4, LMP-1, LMP-2a and LMP-2b. The specific role of EBV in inducing and/or maintaining the neoplastic phenotype of the infected cells in each of these malignancies is not completely understood, although it is known that several of the latent EBV gene products (LMP-1, EBNA-2) are required for B-cell transformation in vitro. LMP-1 leads to constitutive activation of NFkB and upregulation of cellular Bcl-2 in EBV-infected B-cells and thus represents an attractive target for therapeutic intervention. Conversely, lytic EBV gene products have generally been considered to have very limited or no role in B-cell transformation and their expression in clinical specimens of EBV-LPD has not been systematically studied, although drugs inhibiting viral replication (acyclovir, ganciclovir) are frequently used empirically in immunosuppressed patients with EBV-LPD. As the burden of morbidity and mortality due to EBV-LPD is substantial, the identification of novel treatment strategies capable of specifically targeting the EBV-transformed cell is highly desirable.

Over the last several years, we have focused on devising unique strategies to specifically target EBV-LPD, particularly in the context of immunosuppression (IS). Our work thus far has identified several treatment regimens that have produced encouraging results in preclinical animal models and in a
limited number of patients with EBV-LPD. This work has shown that EBV-specific cytotoxic (CD8+) T-cells spontaneously expand in vivo following reduction of IS and that the monitoring of these T-cells over time is helpful to predict outcome in patients with PTLD after kidney transplantation. The observation that a substantial fraction of the expanded CD8+ T-cells was specific for lytic EBV antigens and that most PTLD specimens expressed lytic antigens, such as early lytic gene product viral thymidine kinase (vTk), suggests that, at least in PTLD, lytic EBV antigens may represent a valid therapeutic target, both immunologically and biochemically.

EBV-positive Post-Transplant Lymphoproliferative Disorders (PTLD) respond to reduction of immunosuppression and antiviral therapy

PTLD is a serious complication of allogeneic solid organ and hematopoietic stem cell transplantation that is due to iatrogenic IS and is associated with mortality as high as 70-80%. PTLD is usually EBV-positive, of B-cell lineage, and typically develops in the first 12-18 months after transplantation. More rarely, PTLD may develop late after transplantation (5-10 years), in which case it is more likely to be EBV negative and of T/NK-cell lineage. The incidence of PTLD varies according to the type of allograft, the intensity of IS, and the EBV status of the recipient (EBV seronegative patients having the highest risk). Following solid organ transplantation the incidence ranges from 1-2 percent (kidney transplantation) to 8-10 percent (small bowel transplantation). No standard therapeutic approach exists and the efficacy of reduction of IS and antiviral drugs as front-line therapy for PTLD has remained unclear. We initiated a prospective study looking at a standardized and uniform approach consisting of rapid IS taper and antiviral therapy in patients with EBV-positive PTLD following kidney transplantation, with the goal of determining the response rate to this approach and the immunological basis of response. Immediately after review of the diagnostic biopsy sample and confirmation of the presence of EBV (by EBER1, and 2 or LMP-1 expression), azathioprine or mycophenolic acid were discontinued, the dose of cyclosporin A or tacrolimus was reduced by 50%, and prednisone was quickly tapered to 5-10 mg/day. Patients were simultaneously started on high-dose intravenous acyclovir (10 mg/kg every 8 hours) for 4 weeks, followed by maintenance oral acyclovir (800 mg three times a day) for a total of 12 months. Peripheral blood T-cell subpopulations were measured at baseline and every 4-6 weeks afterwards, peripheral blood mononuclear cells were serially procured to quantitate and analyze EBV-specific T-cells, via HLA-tetramer-peptide analysis. Eleven patients were treated with this approach over 3 years, all with EBV-positive PTLD of diffuse large B-cell lymphoma histology. In all patients examined, CD8+ T-cells expanded rapidly in the peripheral blood after IS taper, with a peak at approximately 10-12 weeks and an increase that ranged from 2.5-fold to 38.2-fold. In most cases the CD8+ T-cell expansion, compared to baseline, was sustained to the time of last follow up. Ten patients achieved a complete response (CR) within a median time of 16 weeks (range 7-26). Of these, nine are in continuous CR, whereas one had a recurrence and was treated with second line therapy (rituximab) (fig. 2). Notably, this patient had a very modest in-

![Figure 2. Clinical outcome in 11 renal transplantation patients with PTLD treated with decreasing immunosuppression and acyclovir. The blue bar represents the time from kidney transplantation to the diagnosis of PTLD. The purple bar represents the time from initiation of therapy to last follow-up, death, or relapse (progression-free survival). Patient no. 2 died of sepsis 4 weeks after diagnosis of PTLD without a documented response. Patient no. 6 relapsed after a 25-month CR with IS taper and acyclovir. A second CR was achieved after withdrawal of IS and institution of rituximab therapy. A second relapse was then treated with high-dose antiviral therapy (azidovudine and ganciclovir), which led to a third CR (clinical, radiologic, and virologic).](image-url)
crease of CD8+ T-cells and was one of the two in whom the expansion following IS taper was not sustained. In patients carrying HLA-class I alleles compatible with available HLA-tetramers for EBV, this technique was used to measure changes in peripheral blood CD8+ T-cells specific for two immunodominant EBV peptides RAK and FLR. RAK and FLR are immunodominant peptides for the early antigen BZLF1 and for the latent gene EBNA-3A, respectively. As shown in figure 3, a population of RAK-specific T-cells expanded remarkably after IS taper in these patients, as they proceeded to clear their PTLD and achieve a CR. FLR-specific CD8+ T-cells did not change (fig. 3B). Interestingly, evidence of lytic EBV activity was found in the majority of PTLD specimens, by in-situ-RT-PCR for the viral gene thymidine kinase. This study demonstrates that kidney transplant patients have a profound suppression of T-cell mediated immunity at the time of development of PTLD and that controlled IS taper results in a remarkable recovery of the number of CD8+ T-cells within 10-12 weeks. This recovery appears to be associated with a high number of complete clinical responses, even in patients with histologically malignant PTLD (DLBCL), where the efficacy of a conservative therapeutic approach has typically been judged very low. The specificity of the expanding CD8+ T-cell population in our study was predominantly directed at the lytic EBV antigen BZLF1. This is interesting given the close relationship between elevated peripheral blood viral DNA load and emergence of PTLD. Although the significance of this observation from the standpoint of clinical response remains unclear, it may be an indication that EBV viral replication in PTLD occurs more frequently than previously recognized and provides an additional rationale to further study the role of virus-directed therapies in this disease (see section C).

Animal models of EBV-LPD and preclinical development of novel therapies

A major focus of our laboratory involves the study of the pathogenesis, prevention and treatment of EBV-LPD in animal models. Much of this work has utilized a xenogeneic (human-murine) preclinical, in vivo model where severe combined immune deficient (SCID) mice are engrafted with human peripheral blood leukocytes (hu-PBL) from healthy human donors who are seropositive for EBV. Following engraftment of hu-PBL (determined by human Ig levels), animals spontaneously develop human EBV-LPD which allows us to examine the pathogenesis of EBV-LPD as well as test the efficacy of various experimental therapeutic strategies in vivo. These tumors...
activation of human memory EBV-antigen specific T cells and that expansion of antigen specific T cells could be enhanced in the presence of human anti- gen-presenting cells (APCs). To promote engraftment or differentiation of human APCs in vivo, we treated mice with human Granulocyte-Monocyte Colony-Stimulating Factor (GM-CSF), a cytokine that promotes differentiation and maintenance of dendritic cells. We hypothesized that treatment of hu-PBL-SCID mice with GM-CSF and low dose IL-2 would promote the outgrowth of human EBV-specific T cells and improve survival. Hu-PBL-SCID mice were randomized to either receive PEG-IL-2, GM-CSF, placebo, or combined GM-CSF and PEG-IL-2 therapy. All mice were depleted of murine natural killer cells with weekly injections of asialo-GM-1 anti-sera. figure 4 shows a survival curve illustrating the significant improvement in survival of mice receiving combined GM-CSF and low dose IL-2 compared to low dose IL-2 alone (p < 0.0041).

There were no significant differences in survival between animals receiving GM-CSF alone, placebo, or PEG-IL-2 alone. Subsequent lymphocyte depletion experiments were performed that identified human NK cells, CD3+/CD8+ T cells and human monocytes as essential mononuclear subsets required for the protective effect of combined GM-CSF and IL-2 therapy.

To further our understanding of events taking place in vivo, we performed flow cytometric studies to evaluate for the presence of human lymphocyte subsets engrafted in hu-PBL-SCID mice at week 5. figure 5 demonstrates the T cell and B cell immunophenotypes using flow cytometric analysis of spleens from these mice. Mice receiving low dose IL-2 had a high percentage of human B cells (Top panel, CD45+/CD19+) compared to mice receiving GM-CSF and IL-2 (lower panel, CD45+/CD19+). PEG-IL-2-treated mice also exhibited lower numbers of human T cells (Top panel, CD3+/CD8+) compared to mice receiving GM-CSF and IL-2 (lower panel, CD3+/CD8+).

To determine the specificity of engrafted human T cells for EBV, we utilized MHC Class I tetramers loaded with EBV peptides. We identified EBV-specific T cells in animals treated with combined GM-CSF and IL-2 but not in animals treated with IL-2 alone (fig. 6). T cells identified were labeled with MHC Class I tetramers (HLA-B8) folded with either FLRGRAYGL or RAKFKQLL immunodominant peptides from the EBV genes products EBNA-3A and BZLF-1 respective- ly. Remarkably, a large fraction of EBV-specific T cells could be enhanced in the presence of human anti- gen-presenting cells (APCs).
prognosis with median survival times of 2-12 months despite aggressive management with radiation therapy. We have developed a pre-clinical model of EBV+ PCNSL to explore strategies that specifically target EBV-infected B lymphoblasts in vivo. Stereotactic implantation of EBV-transformed human lymphoblastoid B cell lines (LCL) into the caudate nucleus of the nude rat resulted in lethal CNS tumor burden manifested by the onset of focal neurological symptoms within 21 days. Radiation (1600 cGy) of LCLs resulted in upregulation of the EBV thymidine kinase transcript and sensitization of these cells to drug-induced apoptosis using the nucleoside analogs AZT and ganciclovir (GCV). In vivo trials using the nude rat PCNSL model demonstrated significantly improved mean survival time (MST) with single fraction whole-brain radiotherapy (WBRT) and AZT/GCV (MST 41.3 ± 3.3 days, p = 0.05), compared to AZT/GCV (MST 32.1 ± 1.1 days) or WBRT alone (MST 22 ± 0.8 days). We discovered constitutive and abundant EBV-TK mRNA expression in a stereotactic core biopsy specimen from a solid organ transplant patient with EBV+ PCNSL (fig. 7). Withdrawal of immunosuppression did not result in disease regression. This patient achieved a complete response after therapy with high dose AZT and GCV in the absence of WBRT (fig. 7), and remains in remission on oral maintenance AZT/GCV therapy 3 years after diagnosis.
These results suggest that anti-viral therapies can be effectively explored in vivo using a pre-clinical animal model of human EBV+ PCNSL, with subsequent translation to patients with EBV+ PCNSL.

**Conclusions**

EBV-related malignancies remain an exciting and fruitful area of basic and translational investigation. These disorders affect very large and heterogeneous groups of patients worldwide and basic discoveries about mechanisms of disease and therapeutic targets are likely to have major public health impact. One of the current challenges is the definition of the cellular interactions and signaling pathways that are unique to EBV-positive malignancies and are critical to induce and sustain the malignant phenotype. The specific road to various EBV-induced malignancies in different patient populations is likely to depend, among other things, on genetic background, immune status, coexisting viral pathogens, and carcinogenic triggers. However, the presence of EBV in the tumor cells provides in principle a unique “Achilles’ heel”. Future research should increasingly focus on the identification of pharmacologically and/or immunologically relevant targets to be exploited for therapy across the spectrum of EBV-induced malignancies.

**References**

A) Stereotactic core biopsy from primary CNS lymphoma demonstrated EBV B-cell lymphoma with evidence of EBV-TK gene expression by in situ RT-PCR. B) Brain MRI from patient at diagnosis and C) after therapy with combination AZT/GCV.


