

The Tumor Microenvironment 4: Drug Resistance in Tumors

A. Background

- 1] historically, the most common indications for the use of chemotherapy include:
 - a) as the major curative modality for some types of tumors, especially hematological ones
 - b) as an adjuvant treatment before or after local therapy for the primary tumor, with the goal of eradicating presumably present, yet possibly still undetectable, metastatic disease
 - c) in sequence or concurrent with radiotherapy, in an attempt to improve the therapeutic gain (e.g., using a chemotherapy agent as a radiosensitizer)
 - d) as a palliative treatment either alone or in combination with other modalities, for many kinds of advanced cancers

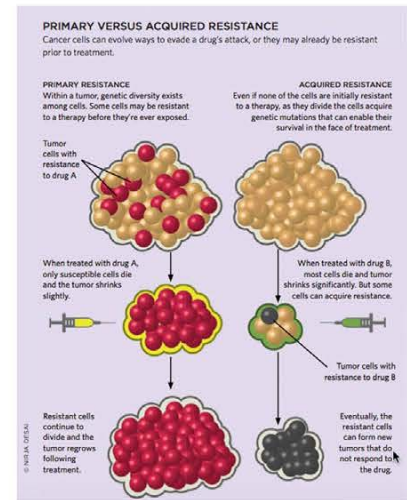
2] despite all these indications for chemotherapy, and its widespread use, the fact remains that many types of cancer show infrequent, erratic or dwindling responses to treatment with anticancer drugs, and the development of drug resistance is thought to be one of the major explanations for this (and, the newer molecularly-targeted drugs are unfortunately no exception)

B. Types of Drug Resistance

Acquired Resistance: a drug-resistant phenotype that develops in response to exposure to a drug

Intrinsic Resistance: a drug-resistant phenotype – as a consequence of genetic and/or epigenetic changes – of a subset of tumor cells that pre-dates exposure to a drug

Extrinsic Resistance: an apparent drug resistance that is not a cellular property *per se*, but that results from microenvironmental conditions in and around tumors



1] there is good evidence that many (if not most) types of drug resistance are intrinsic that is, that drug-resistant, "mutant" cells are already present in tumors prior to drug treatment, and are gradually selected for by repeated drug treatments; this idea was first proposed for mammalian cells by Ling (1982) and Goldie and Coldman (1984)



Illustrating the idea of how a tumor becomes more heterogeneous as it evolves, owing to genomic instability. As such, a drug designed to kill cells of the "blue phenotype" will only enrich the population with the red, orange, green and purple cells (and any new variants that keep appearing). Ideally then, you'd either need to use a drug that kills all the variants, or else use multiple drugs that target specific variants.

a) implications of the Goldie and Coldman hypothesis for the planning of a course of chemotherapy:

1. if the spontaneous mutation theory is correct, then the likelihood of a particular tumor containing at least one drug resistant cell increases with increasing number of tumor cells, and is therefore very high (nearly 100%) even in a "small" tumor containing 10^9 or so cells

a] given this, the best chance to cure a tumor with chemotherapy is when it is as small as possible, that is, early in its natural history

b] to try to reduce the selection pressure toward the drug resistant tumor cells, the best therapeutic effect would be anticipated when different schedules of two equally effective and non-cross resistant drugs are alternated, rather than given in sequence

4] the drug resistant phenotype is just that – resistance to one or more chemotherapy drugs – but what about the genotype of drug resistant cells?

a) the types of genetic lesions most associated with drug resistance are point mutations and gene amplification

1. a point mutation represents a single change (that might be very subtle, such as the change in a single DNA base) that occurs in a single step, with a resulting stable change in phenotype, i.e., from "sensitive" to "resistant"

2. gene amplification occurs in a stepwise manner over time, and drug-resistant cells can be selected more rapidly by exposing cells to gradually increasing concentrations of the selecting drug; also, the resistant phenotype is more likely to be variable, i.e., "slightly resistant" becoming "more resistant" becoming "very resistant"

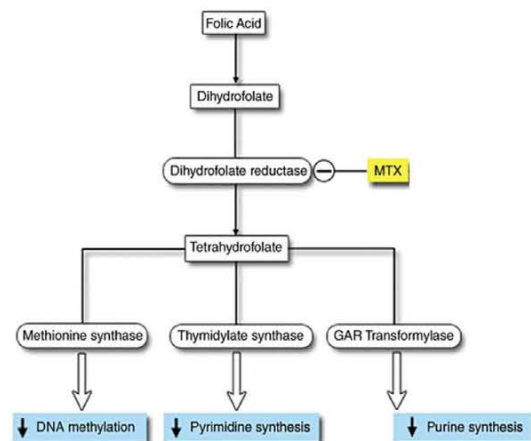
C. Examples of Intrinsic Drug Resistance

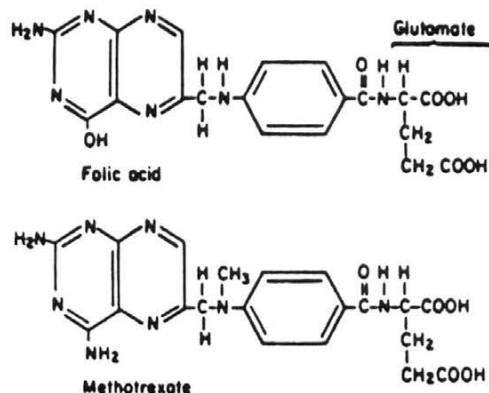
1] Methotrexate Resistance:

a. the most prevalent mechanism leading to methotrexate resistance in cell lines is gene amplification

(1) the resistance is due to overproduction of the enzyme dihydrofolate reductase (DHFR), the target enzyme of methotrexate, which functions in getting the nucleotide bases ready for DNA synthesis [thus, methotrexate's cytotoxicity results from its ability to poison DHFR, and therefore mess up DNA synthesis as well as kill S-phase cells]

Influence of methotrexate on cellular metabolism. Through competitive inhibition of the enzyme dihydrofolate reductase, the drug depletes the pools of reduced folates (FH_4):5,10-methylene tetrahydrofolate ($5,10\text{CH}_2\text{FH}_4$) and 10-formyltetrahydrofolate (10CHOFH_4). Derivatives of these reduced folates are required for purine synthesis and in the conversion of deoxyuridine monophosphate (dUMP) to thymidine monophosphate (dTMP). Interruption of these processes leads to inhibition of DNA synthesis.

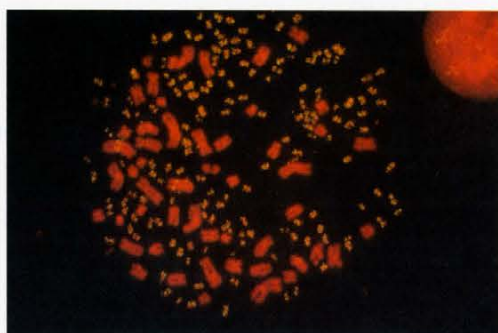




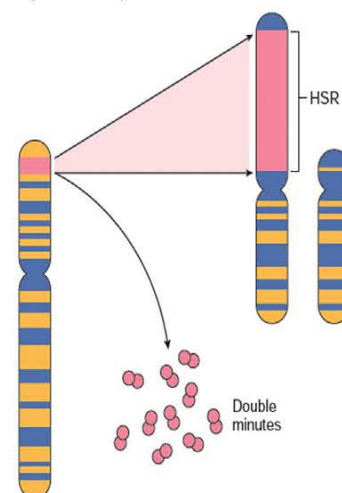
The structure of folic acid, and its analogue methotrexate. Note that glutamate forms one end of these molecules, and further glutamic acid molecules may be added to methotrexate within the cell.

(2) DHFR is overproduced because its gene is amplified--a lot--not necessarily because the gene is transcribed any faster

(3) the extent to which gene amplification occurs can be observed by examining chromosome spreads from methotrexate resistant cells in culture or tumors; these cells show cytogenetic abnormalities called "**double minutes**" and/or "**homogeneous staining regions**" (HSR's)



When DNA is inappropriately amplified, the amplified DNA can often be seen as pairs of tiny chromosomes called double minutes (left) or as expanded, homogeneously staining regions of chromosomes (right). In both cases, the amplified segments are stained yellow.



b. other types of chemotherapy agents, particularly those whose mechanisms of action are similar to methotrexate (i.e., they interfere with DNA synthesis and kill S-phase cells, like 5-fluorouracil), also tend to develop drug resistance through gene amplification

2] "**Multidrug Resistance**" - a different mechanism of drug resistance than mere gene amplification

a. the phenomenon of multidrug resistance was first identified when it was observed that cells selected for resistance to one drug sometimes showed cross-resistance to other drugs of a similar type or class, even though the chemical structures of the other drugs may be quite different

Family of Drugs Associated with Cross-Resistance and Expression of P-glycoprotein

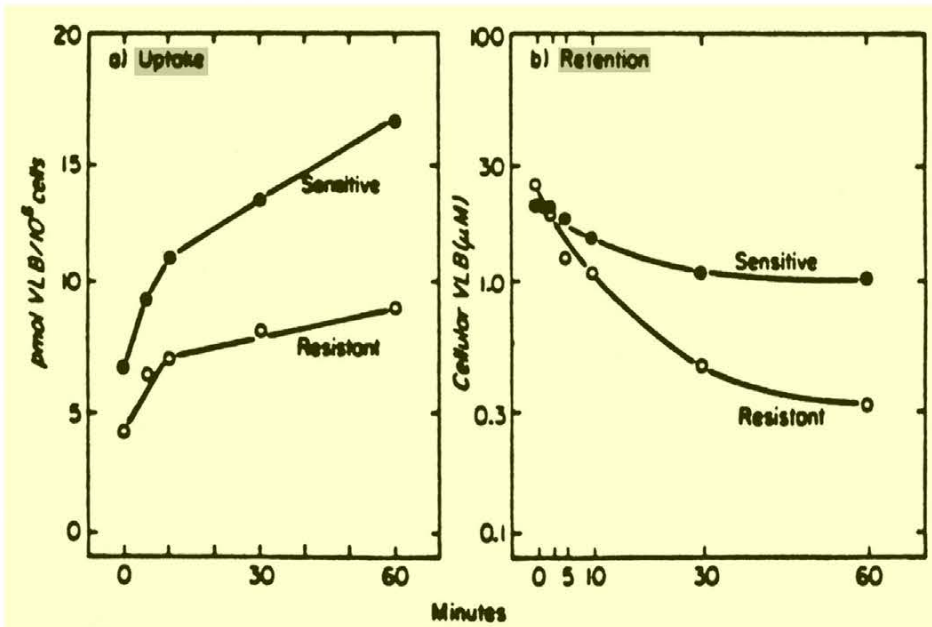
Actinomycin D	Podophyllotoxin
Colchicine	Puromycin
Daunorubicin	Vinblastine
Doxorubicin	Vincristine
(Adriamycin)	Vindesine
Emetine	Melphalan
Etoposide (VP-16)	(weaker association)

(1) in such multidrug resistant cells, *the highest degree of resistance tends to be toward the original drug used for selection*, so the mechanism of resistance is at least somewhat specific

(2) *drugs that are derived from natural products (i.e., plant toxins etc.) account for most of the cases of multidrug resistance*

b. by studying multidrug resistant cells, the following mechanisms were identified:

(1) *such cells have shown both decreased uptake of the drugs they are resistant to, as well as increased efflux of drug that does get into the cells*

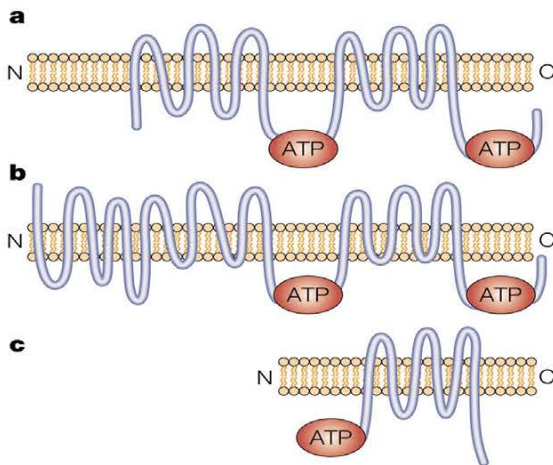


A comparison of vinblastine uptake and efflux in drug sensitive versus multidrug resistant human leukemia cells

(2) *such cells also show increased levels of an integral membrane glycoprotein, whose molecular weight is 170 kd---this has been called the "p-glycoprotein"*

a) it has since been learned that *p-glycoprotein acts as an energy-dependent, membrane "pump" or "transporter"*, which regulates the cellular influx and efflux of drugs; p-glycoprotein is but one member of a large family of membrane-bound transporter proteins referred to collectively as ABC transporters

Structure



Examples

MDR1 (ABCB1)
MRP4 (ABCC4)
MRP5 (ABCC5)
MRP7 (ABCC1)
BSEP/SPGP (ABCB11)

MRP1 (ABCC1)
MRP2 (ABCC2)
MRP3 (ABCC3)
MRP6 (ABCC6)

MXR/BCRP/ABC-P
(ABCG2)

Boards Question: *The gene that codes for the p-glycoprotein is called MDR1, and it is but one of a family of genes that all code for trans-membrane pumps or transporters that require energy (ATP) to operate effectively.*

Structures of ABC transporters known to confer drug resistance. The structures of three categories of ABC transporter. **a** | ABC transporters such as multidrug resistance MDR1 and multidrug-resistance-associated protein 4 MRP4 have 12 transmembrane domains and two ATP-binding sites. **b** | The structures of MRP1, 2, 3 and 6 are similar in that they possess two ATP-binding regions. They also contain an additional domain that is composed of five transmembrane segments at the amino-terminal end, giving them a total of 17 transmembrane domains. **c** | The 'half-transporter' ABCG2 contains six transmembrane domains and one ATP-binding region — in this case, on the amino-terminal side (N) of the transmembrane domain. In other 'half-transporters', such as the transporter associated with antigen processing (TAP), the ATP-binding cassette is found on the carboxy-terminal (C) side of the transmembrane domain. Half-transporters are thought to homodimerize or heterodimerize to function.

(3) ABC transporters, including p-glycoprotein, are normally-occurring membrane proteins that are prevalent in normal tissues likely to encounter environmental toxins (such as from food), so it makes sense that they protect cells by pumping out such toxins; the problem occurs when tumor cells, due to their tissue of origin and/or genetic instability, start to over-express or amplify ABC transporter genes and activate the efflux pumps in response to chemotherapy - then, such cells unfortunately develop the drug resistant phenotype

MDR1 expression in normal and tumour tissue			
Tissue	MDR1 expression		
	High	Intermediate	Low
Normal	Adrenal gland Kidney medulla	Liver Lung Small intestine Colon	Brain Prostate Skin Bone marrow Ovary Stomach
Tumour	Phaeochromocytoma Adrenal Colon Kidney Liver Pancreas Carcinoids		Leukaemia Breast Ovary Thyroid Neuroblastoma Sarcoma Non-Hodgkin's lymphoma

Targeting Multidrug Resistance as a Clinical Strategy?

1. this has been of longstanding interest since the 1980's, when MDR1/p-glycoprotein was first identified and characterized

a) one drug tested early on was **verapamil**, which in some studies improved chemotherapy response in patients who had already developed multidrug resistance, *however the drug had dose-limiting side effects secondary to poisoning ABC transporters in normal tissues*

2. today, there's renewed interest in this area of research, once it was determined that **cancer stem cells upregulate ABC transporters, which is one way (along with growing slowly) they become treatment resistant**

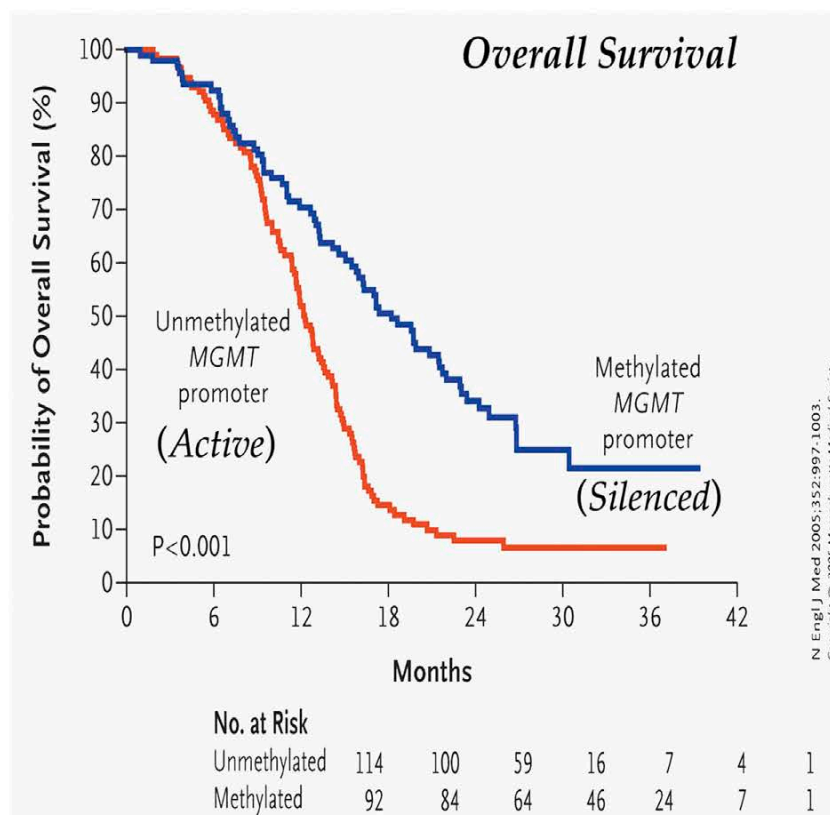
a) newer approaches to accomplishing this include:

- new classes of allosteric, competitive inhibitors that bind to the active sites in ABC transporters
- targeting signaling pathways that cause upregulation of ABC transporters in the first place
- miRNA therapy to shut down expression of MDR-related genes in cancer stem cells
- gene therapy to *increase* the number ABC transporters in normal tissues
- drug delivery using nanoparticles, which are less likely to get pumped out of cells

3] **Temozolamide Resistance in Glioblastoma** - this type of resistance is **epigenetic** in origin, i.e., in which the level of activity of a DNA repair-related gene determines whether the tumor cells are sensitive or resistant to the drug

a. *the gene MGMT (O⁶-methylguanine–DNA methyltransferase) codes for a protein that can repair the types of DNA damage produced by temozolamide...mostly, bulky DNA adducts*

1) if this gene is active, glioblastoma cells are relatively resistant to temozolamide, so there wouldn't be much advantage to using it clinically; in fact, even without adding the drug, activity of this gene is already associated with more aggressive tumors and poorer overall survival



2) however, if the MGMT promoter is hypermethylated, the gene is silenced, and then tumor cells become more sensitive to temozolamide

Overall survival in glioblastoma patients treated with radiotherapy and temozolamide, as a function of whether the MGMT gene is active or silenced.

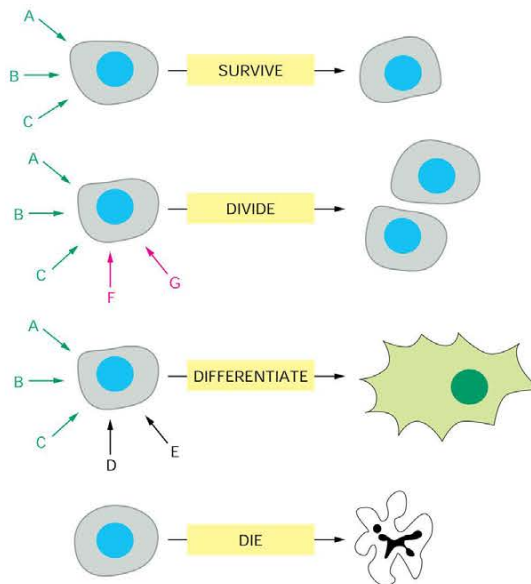
When the gene is epigenetically silenced, the drug is more effective and overall survival is (somewhat) improved.

4) Resistance to Molecularly-Targeted Drugs - the mechanisms of resistance to targeted agents can be intrinsic or acquired, and this depends on what, exactly is being targeted

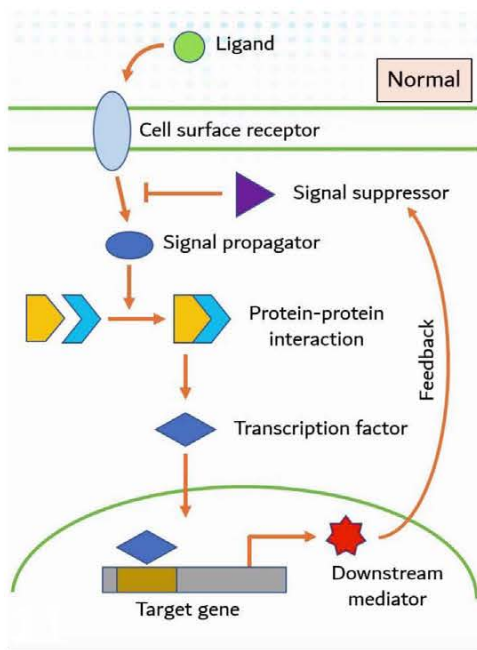
a. *in order to understand how resistance develops, it's first necessary to understand what is being targeted, why and how...*

1] **molecularly targeted drugs target components of cell signaling pathways, usually in an attempt to shut them off** (because mutations have caused them to be stuck in the “on” position)

2] signaling pathways control all the important “decisions” cells have to make during their lifetimes, such as:



3] presently, the majority of molecularly-targeted drugs are trying to turn off proliferate/divide signaling pathways, a major type being those initiated by **receptor tyrosine kinases**



Under normal conditions, an appropriate ligand (e.g., a growth factor) binds to the receptor tyrosine kinase, which then phosphorylates a signal propagator, which then sets off a cascade of protein interactions.

This culminates in the activation of transcription factors that turn on genes related to proliferation (and many other things) that keep the cell alive and functioning.

Note that these pathways also contain suppressor proteins that can work at various points in the signaling cascade to shut off the signal when it's no longer needed.

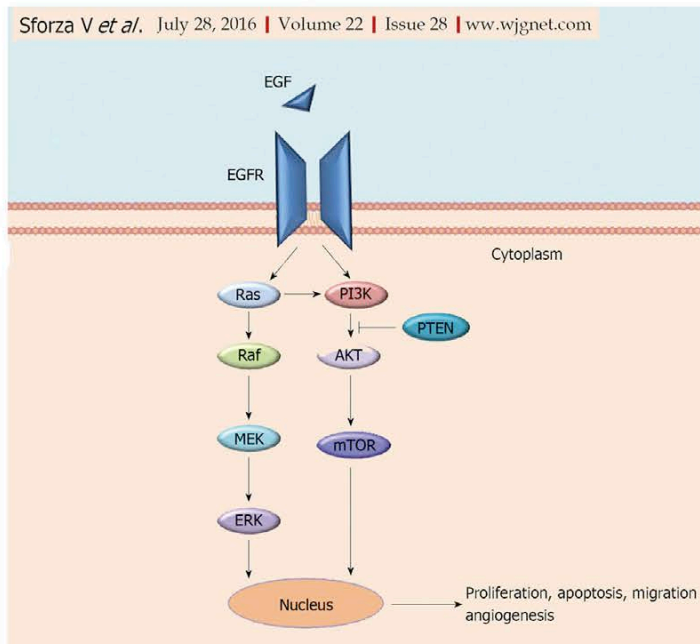
The problem is that in tumor cells, many of these pathways stay on constitutively due to mutations in the signaling components, or else because the shut off proteins are missing or defective.

b. for the purposes of this lecture, we'll focus on **epidermal growth factor receptor (EGFR)** as a representative receptor tyrosine kinase (others include VEGFR, PDGFR, FGFR, HER2, IGFR, etc.) and the signaling pathways under its control

1] many kinds of tumors have defects in the EGFR pathway, which is why it has been a long-standing target of drug development

EGFR Expression in Human Tumors		
EGFR expression		High expression generally associated with
<ul style="list-style-type: none"> • NSCLC 40-80% • Prostate 40-80% • Head & Neck 90-100% • Gastric 33-74% • Breast 14-91% • Colorectal 75-89% • Pancreatic 30-95% • Ovarian 35-77% • Bladder 31-72% • Glioma 40-63% 		<ul style="list-style-type: none"> • Invasion • Metastasis • Late-stage disease • Chemotherapy resistance • Poor outcome

2] receptor tyrosine kinases like EGFR control two major signaling pathways, the **Ras-Raf-MEK-ERK** and the **PI3K-AKT-mTOR** pathways



These are pro-survival pathways, so would encourage things like growth, proliferation, metabolic reprogramming and angiogenesis, and discourage cell death (by apoptosis especially)

Much research and drug development has focused on the Ras-Raf-MEK-ERK pathway, so the issues are better understood in terms of the development of drug resistance.

However work is also being done on targeting the PI3K-AKT-mTOR pathway (but there are fewer drugs approved for clinical use thus far).

a) the key to coping with resistance that develops to drugs that target the EGFR pathway is to understand the different ways that tumor cells could respond to the inhibition of one pathway component, and have a Plan B, C and D (or all at once) ready and waiting...because once resistance to the initial drug occurs, some tumors then progress rapidly

1. When cancer cells overexpress EGFR (either by having too many copies, or by being stuck in the “on” position), the entire pathway gets overstimulated...**meaning that a logical clinical strategy would be to target the EGFR receptor with drugs that will shut it down**

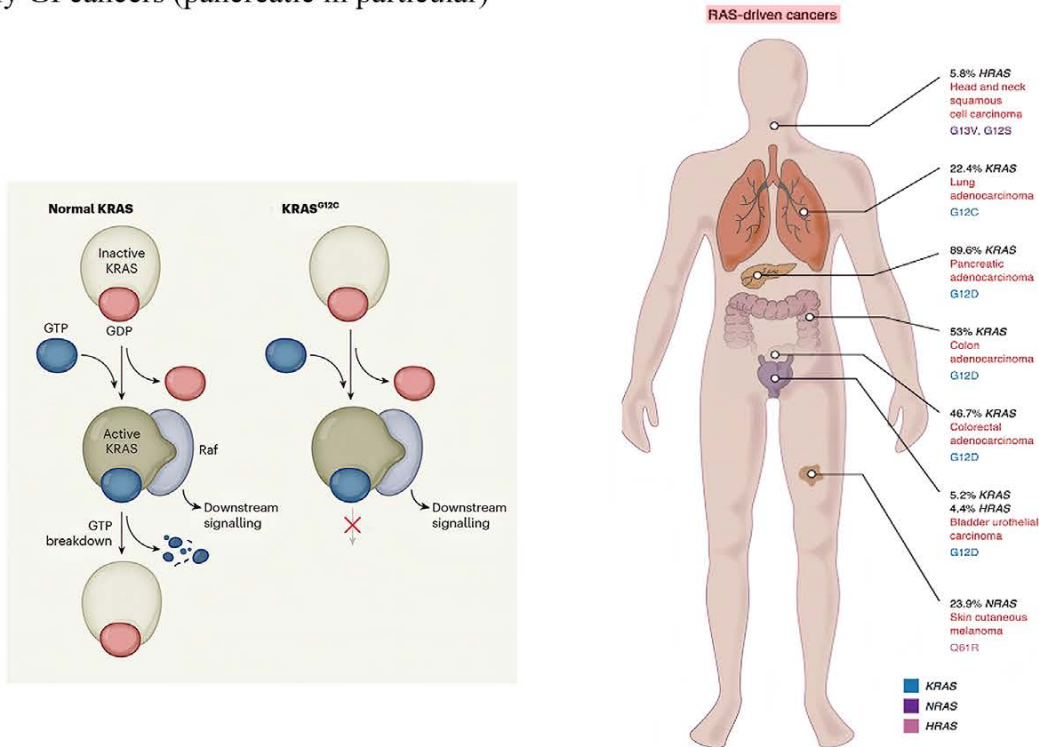
a) this is exactly what drugs like **cetuximab** and **panitumumab** (and others) do, however some tumors don't respond at all, or else they respond initially and then become drug resistant. WHY and HOW?

b) frequently, the issue is that Ras, the next step in the signaling cascade, is also mutated and permanently turned on, such that inhibiting the receptor itself would have no effect

2. OK, so what about targeting RAS (and its variants)?

a) for 30 years, trying to target RAS had been a holy grail of sorts, because based on its 3-dimensional protein structure, it appeared to be “undruggable”

1] research continued however, given how many types of tumors are driven by mutant forms of RAS, especially GI cancers (pancreatic in particular)



N ENGL J MED 383:13 NEJM.ORG SEPTEMBER 24, 2020

	NSCLC (N=59)	Colorectal Cancer (N=42)	Other (N=28)
Best overall response — no. (%)			
Confirmed complete response	0	0	0
Confirmed partial response	19 (32.2)	3 (7.1)	4 (14.3)
Stable disease	33 (55.9)	28 (66.7)	17 (60.7)
Progressive disease	5 (8.5)	10 (23.8)	4 (14.3)
Could not be evaluated	1 (1.7)	0	1 (3.6)
No assessment*	1 (1.7)	1 (2.4)	2 (7.1)
Objective response — % (95% CI)†	32.2 (20.62–45.64)	7.1 (1.50–19.48)	14.3 (4.03–32.67)
Disease control — % (95% CI)‡	88.1 (77.07–95.09)	73.8 (57.96–86.14)	75.0 (55.13–89.31)

* One patient with NSCLC withdrew consent before tumor assessment. One patient with colorectal cancer and 2 patients with other tumor types had clinical progression.

† Objective response was defined as a complete or partial response.

‡ Disease control was defined as a complete response, a partial response, or stable disease.

At long last, a new drug has arrived (and others are in the pipeline): **sotorasib** (Lumakras®). It specifically targets the **KRAS G12C** mutation, which is found in about 13% of NSCLC patients, and is also being evaluated in other sites in (small-ish) Phase I and II clinical trials.

Sotorasib produced objective responses in about a third of NSCLC patients bearing the mutation, although considerably less so in colorectal cancer and others.

Toxicity is also an issue, but at least it's a start!

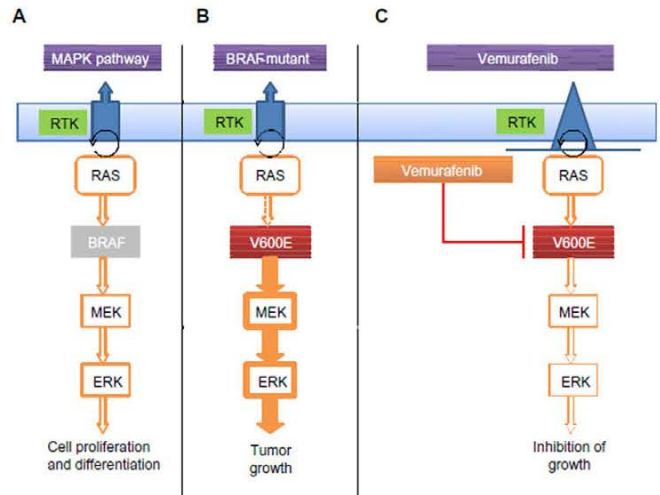
c) Next step? **Drugging Raf**, the next link in the signaling chain - better luck here than for Ras!

1] Case in point: **B-Raf mutant melanoma**

In some melanomas, activating mutations in BRAF allow the pathway to activate regardless of the status of EGFR.

Thus, focus shifted from inhibiting EGFR to finding drugs that would inhibit BRAF instead.

One such drug, **vemurafenib** (formerly PLX4032), administered to patients with advanced melanoma, produced spectacular and durable tumor regression (in around half the cases)



A 38 year old patient with widespread metastatic melanoma prior to treatment with vemurafenib (A) and after 15 weeks of drug therapy (B).

Amazing!

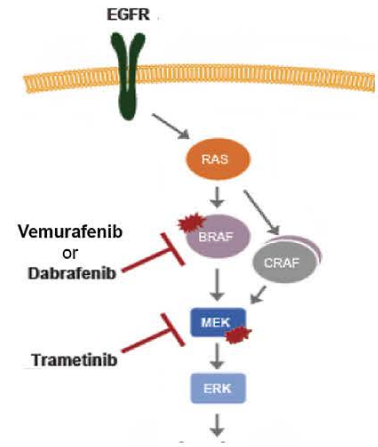
(Melanoma cells treated with vemurafenib die by apoptosis)



But, after 23 weeks of treatment with vemurafenib, the melanoma came roaring back with a vengeance.

SO WHAT HAPPENED?

- 1] KRAS starts signaling through CRAF as a means of working around BRAF inhibition
- 2] downstream mutation in MEK, that allow the pathway to remain active regardless of the status of BRAF (or EGFR or RAS) – this was the first example of a new mutation *acquired* in response to inhibition of an upstream player (i.e., this mutation was NOT preexisting and selected for during treatment, but actually “evolved” along the way)



d) What to try next?

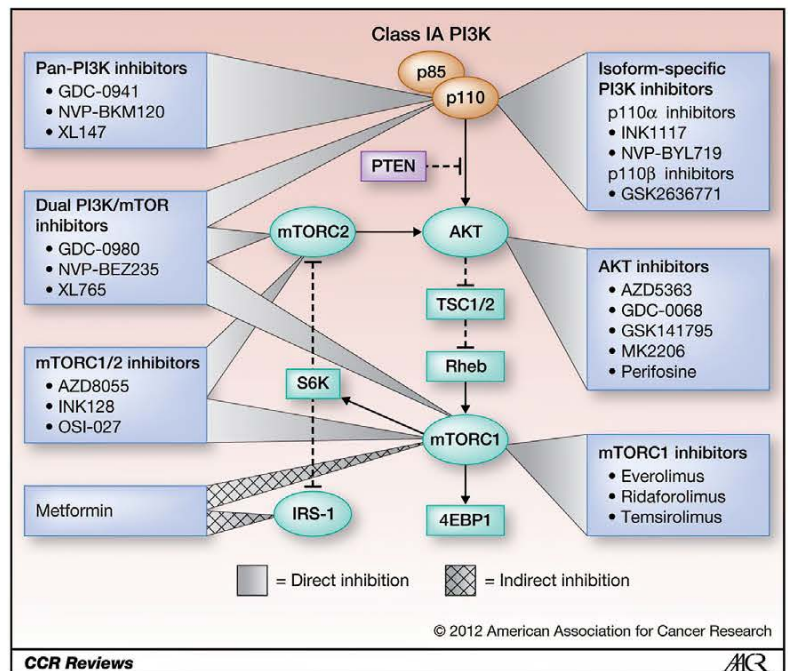
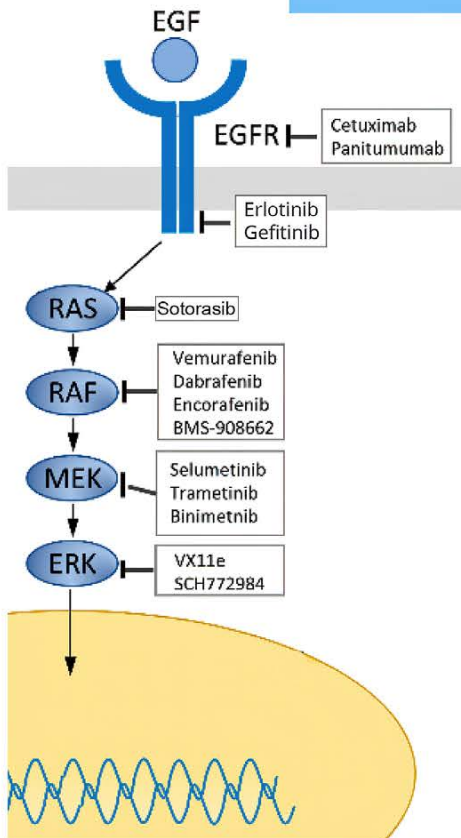
- 1] A MEK inhibitor...such as **cobimetinib** or **trametinib** (typically used in combination with vemurafenib or dabrafenib)
- 2] another case in point: **metastatic colorectal cancer with defects in EGFR signaling**

a. Does targeting EGFR, mutant Ras or mutant B-Raf produce the same results in colorectal cancer as in melanoma?

Answer: NO, which just goes to show that the behavior of these signaling pathways depends on the exact context, and therefore can be tissue specific

Less than 10% of patients respond (compared to ~50% of patients with melanoma), and in some cases, the addition of vemurafenib or darefenib makes matters worse.

Summary: Drugging the EGFR Pathway



J Clin Oncol 29:3085-3096. © 2011

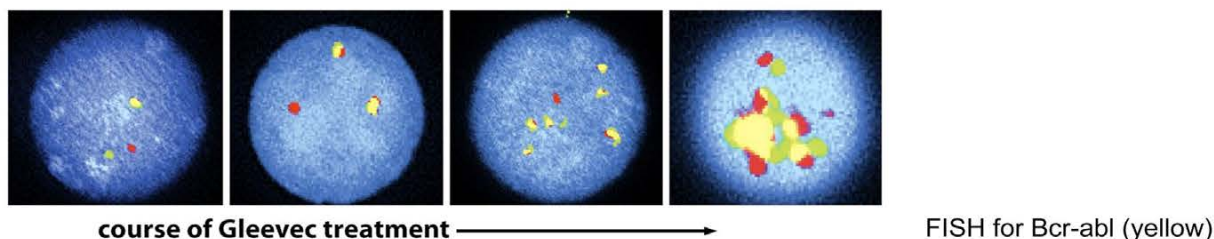
b. Example 2: **Imatinib Resistance**

1) *Imatinib is used to treat CML by inactivating the mutant kinase fusion protein BCR-ABL, and to treat GIST by blocking aberrant signaling by onco-protein kinases of mutant c-KIT and PDGFR genes*

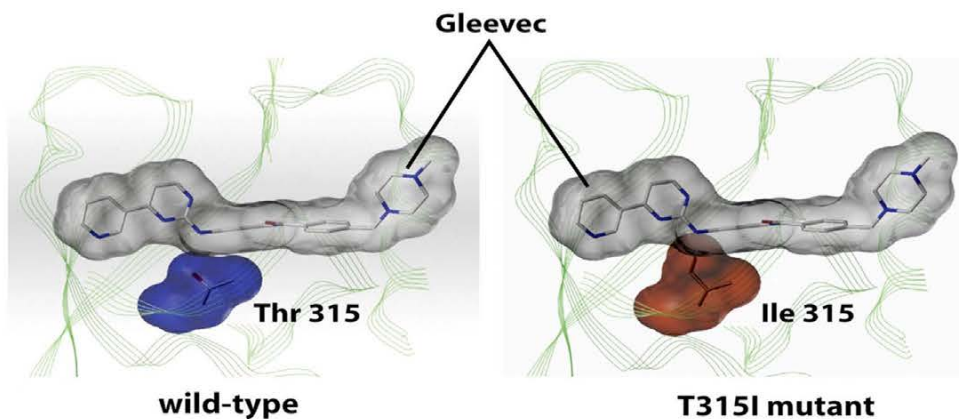
a] Resistance that develops to imatinib is interesting in that there are several different intrinsic mechanisms, any or all of which can confer drug resistance

1. *upregulation and release into the plasma of α 1-acid glycoprotein, which acts to bind imatinib, and reduce its availability to bind BCR-ABL or block c-KIT/PDGFR*

2. *amplification of the BCR-ABL mutant gene*



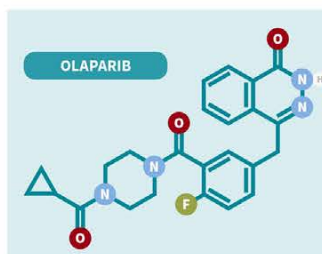
3. *variations in the amino acid sequence of the BCR-ABL protein such that imatinib can no longer bind and inactivate it (from further point mutations in the BCR-ABL gene that could be intrinsic or acquired)*



c. Example #3: **Resistance to PARP Inhibitors**

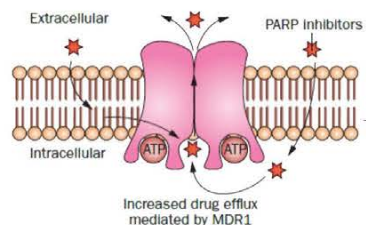
1) *tumor cells have developed several sneaky ways of overcoming PARP inhibition by drugs such as **olaparib**, some fairly straightforward, and others unique*

Olaparib interferes with PARP's ability to sense the presence of DNA SSB's, which interferes with repair, and it also "traps" PARP onto the DNA, which further interferes with repair, and also disrupts DNA replication



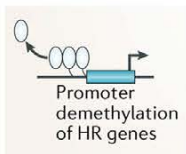
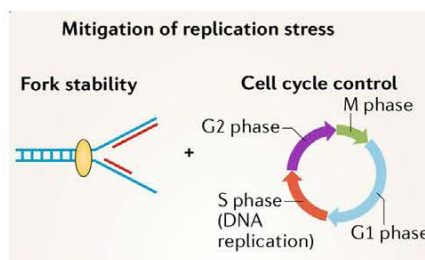
Olaparib is used in patients with BRCA gene defects whose tumor cells already can't repair DNA double stranded breaks through homologous recombination. PARP inhibition "synthetically" produces a second repair defect, in the hopes that this would be sufficient to kill these cells.

a] **“old school” drug resistance can occur through the upregulation of the p-glycoprotein membrane transporter, which pumps olaparib out of the tumor cells**



b] some tumor cells develop PARP inhibitor resistance by **downregulating PARP altogether**, so that it can no longer get trapped on the DNA strand, or else **selection of cells with pre-existing PARP gene mutations that affect olaparib’s PARP binding site**

c] **relief of replicative stress caused by PARP trapping by slowing down the cell cycle**

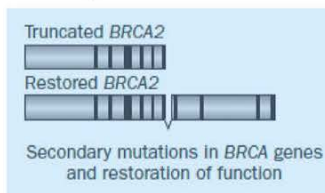


d] **attempt to reactivate homologous recombination!**

(1) demethylate silenced genes associated with HR

(2) downregulate protein 53BP1; it encourages the cell to repair DSBs using NHEJ, so in its absence, the cell will attempt to reactivate HR

(3) (partially) restore the activity of BRCA 1/2 through additional acquired gene mutations or chromosomal rearrangements



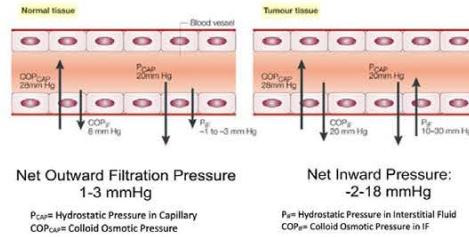
D. Extrinsic Mechanisms of Drug Resistance

1] **too high a tumor cell burden** - if a tumor isn’t diagnosed before it has become too large, it would be nearly impossible to cure within the constraints of normal tissue tolerance, even under the best of circumstances (another argument for early detection!)

2] **inaccessibility of drugs to all tumor cells**

a) drug inaccessibility to the tumor can occur for a number of reasons, including:

1. *rapid drug metabolism by intervening cells* (not unlike oxygen consumption); and
2. *high interstitial fluid pressure in tumors*



3. *assorted abnormalities in the tumor's vasculature*
4. *physical stress or stiffness of the tumor's stroma*
5. *pharmacokinetic issues, e.g., a pro-drug doesn't get activated by the liver*

3] anatomical restrictions

a) and in the case of brain tumors, the tumor's vasculature might be sufficient to allow drug access, but the (normal) **blood brain barrier** makes that impossible

A FORMIDABLE BARRIER

The blood-brain barrier (BBB) is a collection of specialized cells and proteins that control the movement of molecules from the blood to the central nervous system (CNS). The blood vessel endothelial cells of the BBB are cemented together by protein structures called tight junctions, preventing diffusion of most molecules between cells. BBB endothelial cells display transporters, receptors, and channels that facilitate selective transport of vital nutrients into the CNS. They also possess efflux pumps, such as P-glycoprotein, that expel most small, amphiphilic molecules that are soluble in the blood and in cell lipid membranes. Pericytes and astrocyte pseudopods serve as an additional physical barrier between the blood vessel and brain tissue, and support the expression of endothelial cell genes required to maintain the BBB.

CONVECTION-ENHANCED DELIVERY
 Catheters placed in the brain through a hole in the skull allow the passage of even large drugs. Pressure is used to infuse a drug as rapidly as possible into a specified region of the brain.

tight junction
 claudin
 occludin

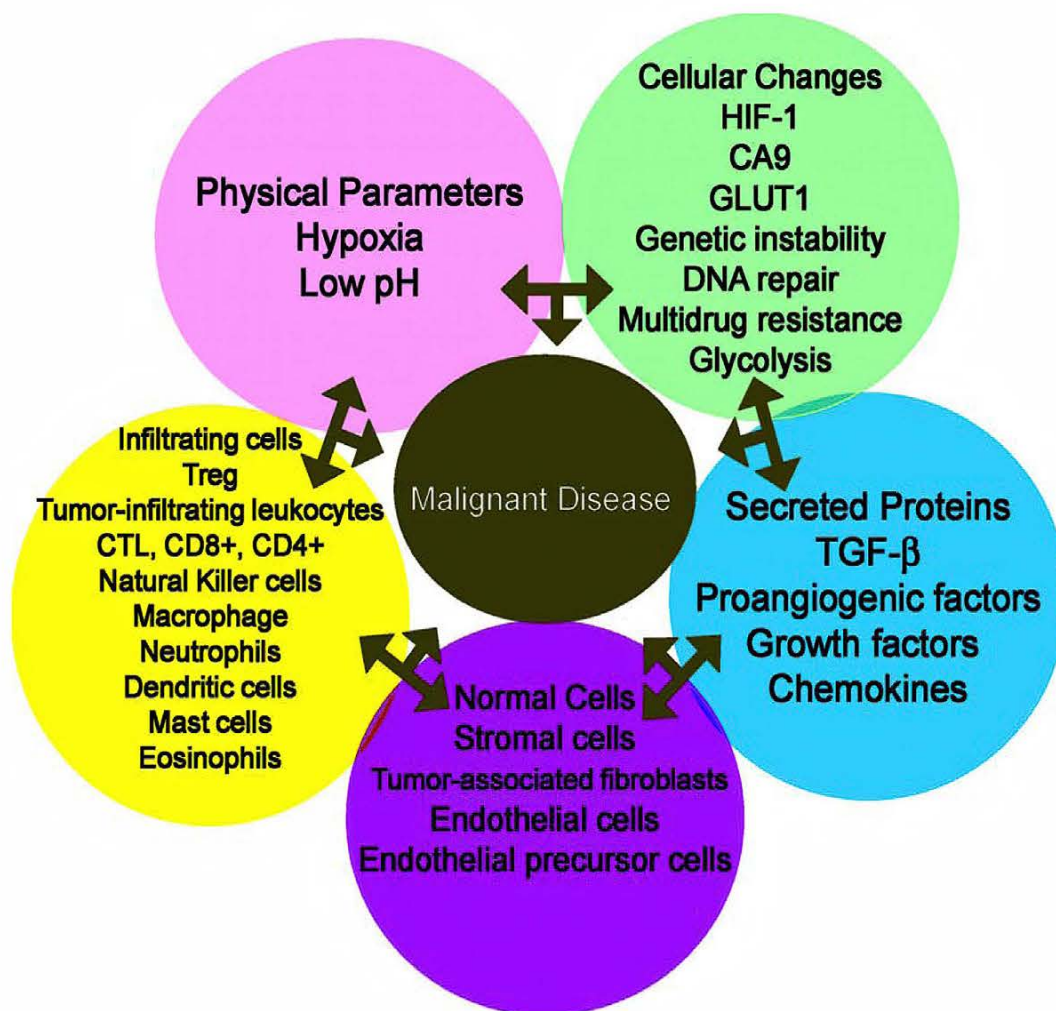
adherens junction
 junction adhesion molecule
 VE-cadherin

Tight junction and adherens junction

Yu X et al. Front Neurosci. 2020

Appendix Materials

Summary Graphic: Drug Resistance - never forget the fact that a tumor is a dynamic, constantly evolving system, and that many of these (and other) possible mechanisms of drug resistance probably coexist and interact with each other...and change over time!



In vivo therapeutic resistance results from an interplay of factors characteristic of the abnormal physiology of malignant tumors.